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9520486.33. Full name, address and postcode of the or of each applicant (*underline all surnames*)

Cambridge Antibody Technology Limited
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 Cambridgeshire
 SG8 6JJ

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

GB

S781992002 AB /

4. Title of the invention

Specific Binding Members for Human Transforming Growth Factor Beta; Materials and Methods

5. Name of your agent (*if you have one*)

Mewburn Ellis

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(including the postcode)

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Patents ADP number (*if you know it*)

109006

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Country

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Number of earlier application

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Claim(s)

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SPECIFIC BINDING MEMBERS FOR HUMAN TRANSFORMING GROWTH
FACTOR BETA; MATERIALS AND METHODS

This invention relates to specific binding members for human transforming growth factor (TGF) beta and materials and methods relating thereto. In particular, it relates to specific binding members comprising antibody binding domains; for example, human antibodies. Human antibodies against human TGFbeta may be isolated and utilised in the treatment of disease, particularly fibrotic disease and also immune/inflammatory diseases. The isolation of antiself antibodies from antibody segment repertoires displayed on phage has been described (A.D.Griffiths et al. EMBO J. 12, 725-734, 1993; A. Nissim et al. EMBO J. 13, 692-698, 1994; A.D. Griffiths et al. 13, 3245-3260, 1994; C.Barbas et al. Proc. Natl. Acad. Sci. USA 90, 10003-10007 1993; WO93/11236).

TGFbeta is a cytokine known to be involved in many cellular processes such as cell proliferation and differentiation, embryonic development, extracellular matrix formation, bone development, wound healing, hematopoiesis and immune and inflammatory responses(A.B. Roberts & M. Sporn 1990 pp419-472 in Handbook of Experimental Pharmacology eds M.B. Sporn & A.B. Roberts, Springer Heidelberg; J.Massague et al. Annual Rev. Cell Biol. 6, 597-646, 1990).

The accumulation of excessive extra-cellular matrix is associated with various fibrotic diseases. Thus there is a need to control agents such as TGFbeta1 and TGFbeta2 to prevent their deleterious effects in such diseases and this is one application of human antibodies to human TGFbeta.

The modulation of immune and inflammatory responses by TGFbetas includes (i) inhibition of proliferation of all T-cell subsets (ii) inhibitory effects on proliferation and function of B lymphocytes (iii) down-regulation of natural-killer cell activity and the T-cell response (iv) regulation of cytokine production by immune cells (v) regulation of macrophage function and (vi) leucocyte recruitment and activation.

A further application of antibodies to TGFbeta may be in the treatment of immune/inflammatory diseases such as rheumatoid arthritis, where these functions

need to be controlled.

It is a demanding task to isolate an antibody fragment specific for TGFbeta of the same species. Animals do not normally produce antibodies to self antigens, a phenomenon called tolerance (G.J. Nossal Science 245, 147-153, 1989). In general, vaccination with a self antigen does not result in production of circulating antibodies. It is therefore difficult to raise human antibodies to human self antigens. There are also in addition, ethical problems in vaccinating humans. In relation to the raising of non-human antibodies specific for TGFbeta, there are a number of problems. TGFbeta is an immunosuppressive molecule and further, there is strong conservation of sequence between human and mouse TGFbeta molecules. Mouse and human TGFbeta1 only differ by one amino acid residue, an alanine (human) to serine (mouse) change at a buried residuc (R.Deryck et al. J.Biol. Chem. 261, 4377-4379, 1986). This makes it difficult to raise antibodies in mice against human TGFbeta. Further, any antibodies raised may only be directed against a restricted set of epitopes.

Polyclonal antibodies binding to human TGFbeta1 and human TGFbeta2 against both neutralising and non-neutralising epitopes have been raised in rabbit (Danielpour et al. Growth Factors 2 61-71, 1989; A. Roberts et al. Growth Factors 3, 277-286, 1990), chicken (R&D Systems, Minneapolis) and turkey (Danielpour et al. J. Cell Physiol. 138, 79-86, 1989). Peptides representing partial TGFbeta sequences have also been used as immunogens to raise neutralising polyclonal antisera in rabbits (W.A Border et al. Nature 346, 371-374, 1990; K.C. Flanders Biochemistry 27, 739-746, 1988). In addition there have been limited reports of isolation of mouse monoclonals against TGFbeta. Following immunisation with bovine TGFbeta2 (identical to human TGFbeta2), three non-neutralising monoclonal antibodies were isolated that are specific for TGFbeta2 and one neutralising antibody that is specific for TGFbeta1 and TGFbeta2 (J.R. Dasch et al. J. Immunol. 142, 1536-1541, 1989). In another report, following immunisation with human TGFbeta1, neutralising antibodies were isolated which were either specific for TGFbeta1 or cross-reacted with

TGFbeta1, TGFbeta2 and TGFbeta3 (C. Lucas et al. J.Immunol. 145, 1415-1422, 1990).

This application discloses the first isolation of human antibodies directed against human TGFbeta1 and against human TGFbeta2.

5 Phage antibody technology (WO92/01047; PCT/GB92/00883; PCT/GB92/01755; WO93/11236) offers the ability to isolate directly human antibodies against human TGFbeta. In application WO93/11236 the isolation of antiself antibodies from phage display libraries was disclosed and it was suggested that antibodies specific for TGFbeta could be isolated from phage display libraries.

10

The present application shows that antibodies of differing specificities for TGFbeta molecules may be isolated. TGFbeta1, TGFbeta2 and TGFbeta3 are a closely related group of cytokines. They are dimers consisting of two 112 amino acid monomers joined by an interchain disulphide bridge. TGFbeta1 differs from TGFbeta2 by 27 mainly conservative changes and from TGFbeta3 by 22 mainly conservative changes. These differences have been related to the 3D structure (M.Schlunegger & M.G.Grutter Nature 358, 430-434, 1992). The present applicants have isolated antibodies which are essentially specific for TGFbeta1 (very low cross-reactivity with TGFbeta2); antibodies which are essentially specific for TGFbeta2 (very low cross-reactivity TGFbeta1); and antibodies which bind both TGFbeta1 and TGFbeta2. Hence, these three different types of antibodies, each type with distinctive binding specificities must recognise different epitopes on the TGFbeta molecules. These antibodies have very low cross-reactivity with TGFbeta3.

25

It has further been demonstrated by the applicants that antibodies specific for TGFbeta can be isolated from libraries derived from different sources of immunoglobulin genes: from repertoires of natural immunoglobulin variable domains; and synthetic repertoires derived from germline V genes combined with synthetic CDR3s. The properties of these antibodies in single chain Fv and whole IgG4 format are described.

3

As noted above WO93/11236 suggested that human antibodies directed against human TGFbeta could be isolated from phage display libraries. The applicants show that the phage display libraries from which antiself antibodies were isolated in WO93/11236 may be utilised as a source of human antibodies specific for human TGFbeta. For instance, in example 1 of the present application, the antibody 1A-E5 specific for TGFbeta1 and the antibodies 2A-H11 and 2A-A9 specific for TGFbeta2 were isolated from the 'synthetic library' described in examples 5 to 7 of WO93/11236 and in Nissim et al. (1994; *supra*). Also, the phage display library derived from peripheral blood lymphocytes (PBLs) of an unimmunised human (examples 1 to 3 of WO93/11236) was the source for the antibody 1B2 specific for TGFbeta1. Phage display libraries made subsequently utilising antibody genes derived from human tonsils and bone marrow, have also provided sources of antibodies specific for human TGFbeta. Thus human TGFbeta is an example of a human self antigen to which antibodies may be isolated from 'large universal libraries'. Human antibodies against human TGFbeta with improved properties can be obtained by chain shuffling for instance combining the VH domains of antibodies derived from one library with the VL domains of another library thus expanding the pool of VL partners tested for each VH domain. For instance, the antibodies 6B1, 6AH and 6III specific for TGFbeta2 utilise the 2A-H11 VH domain isolated from the 'synthetic library' combined with a light chain from the PBL library.

Thus the VII and VL domains of antibodies specific for TGFbeta can be contributed from phage display libraries derived from rearranged V genes such as those in PBLs, tonsil and bone marrow and from V domains derived from cloned germline V segments combined with synthetic CDRs. There are also shown to be a diverse range of antibodies which are specific for TGFbeta1 or TGFbeta2. The antibodies which have been isolated both against TGFbeta1 and TGFbeta2 have mainly utilised V genes derived from VH germlines of the VH3 family. A wider variety of light chain variable regions have been used, of both the lambda and kappa types.

Individual antibodies which have been isolated have unexpectedly advantageous properties. For example, the antibodies directed against TGFbeta2 (6H1, 6A5 and 6B1) have been shown to bind to TGFbeta2 with slow off-rates (off-rate constants k_{off} of the order of 10^{-3} s^{-1}) to neutralise TGFbeta2 activity in in vitro assays and to be potent in in vivo applications. The properties of these antibodies may make them particularly suitable for therapeutic applications. The fact that these antibodies share the same heavy chain, shows that VH domains can be effective with a number of different light chains, although there may be differences in potency or subtle changes of epitope with different light chains.

5 The antibodies directed against TGFbeta1 (1AE5, 1AH6 and 1B2 and their derivatives) also have unexpectedly advantageous properties. Antibody 27C1/10A6 derived from 1B2 by chain shuffling, spiking and conversion into whole antibody IgG4 , has been shown to be potent in an in vitro scarring model. The VH domain of this antibody was derived by site directed 'spiking' 10 mutagenesis from the parent antibody 7A3. A large number of spiked clones were obtained which show similar properties in in vitro assays. There can be a number of changes in CDR3 of the VH compared to 27C1, for instance, 28A-H11 differs in 7 of the 14 positions, 2 of which are non-conservative changes. Thus there 15 may be up to 50% of the residues in the VH CDR3 changed without affecting binding properties.

20 Antibodies specific for human TGFbeta1 and human TGFbeta2 have been shown 25 to be effective in animal models for the treatment of fibrotic diseases and other diseases such as rheumatoid arthritis where TGFbeta is overexpressed. Antibodies against TGFbeta have been shown to be effective in the treatment of glomerulonephritis (W.A Border et al. Nature 346, 371-374, 1990); neural 30 scarring (A. Logan et al. Eur. J. Neurosci. 6, 355-363, 1994); dermal scarring (M. Shah et al. Lancet 339, 213-214 1992; M. Shah et al. J. Cell Science 107, 1137-1157, 1994; M. Shah et al. 108, 985-1002, 1995); lung fibrosis (S.N. Giri et al. Thorax 48, 959-966, 1993); arterial injury (Y.G. Wolf, L.M. Rasmussen & E. Ruoslahti J. Clin. Invest. 93, 1172-1178, 1994) and rheumatoid arthritis (Wahl et al J. Exp. Medicine 177, 225-230, 1993). It has been suggested that TGFbeta3

5 acts antagonistically to TGF β 1 and TGF β 2 in dermal scarring (M. Shah et al. 1995 supra.). Therefore, antibodies to TGF β 1 or TGF β 2 with low cross-reactivity to TGF β 3 as disclosed in this application should be advantageous in this and other conditions such as fibrotic conditions in which it is desirable to counteract the fibrosis promoting effects of TGF β 1 and TGF β 2.

10 There are likely to be applications further to the above mentioned conditions, as there are several other in vitro models of disease where antibodies against TGF β have shown promise of therapeutic efficacy including antibodies directed against TGF β 2 for the treatment of eye diseases such as proliferative retinopathy (R.A. Pena et al. Invest. Ophthalmology. Vis. Sci. 35, 2804-2808, 1994), retinal detachment and post glaucoma drainage surgery. Other diseases which have potential for treatment with antibodies against TGF β include adult respiratory distress syndrome, cirrhosis of the liver, post myocardial infarction, 15 post angioplasty restenosis, keloid scars and scleroderma.

20 The use of antibodies against TGF β for the treatment of diseases has been the subject of patent applications for fibrotic disease (WO91/0478); dermal scarring (WO92/17206); macrophage deficiency diseases (PCT/US93/00998); macrophage pathogen infections (PCT/US93/02017); neural scarring (PCT/US93/03068); vascular disorders (PCT/US93/03795); prevention of cataract (WO95/13827). The human antibodies against human TGF β disclosed in this application should be valuable in these conditions.

25 The applicants show herein that the human antibodies both against human TGF β 1 and against human TGF β 2 can be effective in the treatment of fibrosis in animal models of neural scarring and glomerulonephritis in either single chain Fv and whole antibody format. This is the first disclosure of the effectiveness of antibodies directed only against TGF β 2 as sole treatment in 30 these indications, although some effectiveness of antibodies against TGF β 2 only has been observed in a lung fibrosis model (Giri et al. Thorax 48, 959-966, 1993 supra). The effectiveness of the human antibodies against human TGF β in

treatment of fibrotic disease has been determined by measuring a decrease in the accumulation of components of the extracellular matrix, including fibronectin and laminin in animal models.

5

TERMINOLOGY

10 **Specific Binding Member**

This describes a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other.

Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is concerned with antigen-antibody type reactions.

20 **Antibody**

This describes an immunoglobulin whether natural or partly or wholly synthetically produced.

25 The term also covers any protein having a binding domain which is, or is homologous to, an antibody binding domain. These proteins can be derived from natural sources, or they may be partly or wholly synthetically produced.

Example antibodies are the immunoglobulin isotypes and their isotypic subclasses; fragments such as Fab, F(ab), scFv, Fv, dAb, Fd; and diabodies.

30

Antibody Binding Domain

This describes the part of an antibody which comprises the area which specifically

binds to and is complementary to part or all of an antigen. Thus where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope.

5 An antibody binding domain is provided by one or more antibody variable domains.

Specific

10 This refers to the situation in which a pair of binding members bind only to each other. Thus one member of the specific binding pair will not show any significant binding to molecules other than its specific binding partner.

15 The term is also applicable where eg an antibody binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

Neutralisation

20 This refers to the situation in which the binding of a molecule to another molecule results in the abrogation of the biological effector function of the another molecule.

Functionally Equivalent Variant Form.

25 This refers to a molecule (the variant) which although having structural differences to another molecule (the parent) retains some significant homology and also at least some of the biological function of the parent molecule. Variants may be in the form of fragments, derivatives or mutants.

Substantial Part

30 A molecule may comprise only a part of a sequence referred to. The part sequence will be of sufficient length to substantially retain the function of interest of the full-length sequence.

The present invention provides a specific binding member which comprises a human antibody binding domain specific for TGFbeta1 and/or TGFbeta2 and
5 which has low cross reactivity with TGFbeta3. The TGFbeta may be human TGFbeta.

The specific binding member may be in the form of an antibody fragment such as
single chain Fv (scFv). Other types of antibody fragments may also be utilised
10 such as Fab, Fab', F(ab')₂, Fabc, Facb or a diabody (G.Winter & C.Milstein
Nature 349, 293-299, 1991; WO94/13804). The specific binding member may be
in the form of a whole antibody. The whole antibody may be in any of the forms
of the antibody isotypes eg IgG, IgA, IgE, and IgM and any of the forms of the
isotype subclasses eg IgG1 or IgG4.

15 The specific binding member may also be in the form of an engineered antibody
eg bispecific antibody molecules (or fragments such as F(ab')₂) which have one
antigen binding arm (ie specific binding domain) against TGFbeta and another
arm against a different specificity. Indeed the specific binding members directed
20 against TGFbeta1 and TGFbeta2 described herein may be combined in a bispecific
diabody format. For example the antibodies 31G9 directed against TGFbeta1 and
6H1 directed against TGFbeta2 may be combined to give a single dimeric
molecule with both specificities.

25 The binding domain may comprise part or all of a VII domain encoded by a germ
line gene segment or a re-arranged gene segment. The binding domain may
comprise part or all of either a VL kappa domain or a VL lambda domain.

30 The binding domain may comprise a VH3 gene sequence of one of the following
germ lines; the DP49 germ line; the DP53 germ line; the DP50 germ line; the
DP46 germ line; or a re-arranged form thereof.

The specific binding member may neutralise the in vitro and/or in vivo effect of TGFbeta.

5 The specific binding member may be a high affinity antibody.

The binding domain may comprise part or all of a VH domain having either an amino acid sequence as shown in Fig 1(a)(i) to (iv) or Fig 1(c)(i) or a functionally equivalent variant form of a said amino acid sequence.

10 The binding domain may comprise part or all of a VH domain encoded by either a nucleotide sequence as shown in Fig 1(a)(i) to (iv) or Fig 1(c)(i) or a functionally equivalent variant form of a said nucleotide sequence.

15 The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 1(a)(v) or Fig 1(b) or a functionally equivalent variant form of a said amino acid sequence.

20 The binding domain may comprise part or all of a VL domain encoded by either a nucleotide sequence as shown in Fig 1(a)(v) or Fig 1(b) or a functionally equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a VH domain having a variant form of the Fig 1(a)(i) amino acid, the variant form being one of those as provided by Fig 3.

25 The binding domain may comprise part or all of a VH domain having either an amino acid sequence as shown in Fig 2(a)(i) to (iii),(v) and (vi) or a functionally equivalent variant form of a said amino acid sequence.

30 The binding domain may comprise part or all of a VH domain encoded by either a nucleotide sequence as shown in Fig 2(a)(i) to (iii),(v) and (vi) or a functionally equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 2(a)(iv) or Fig 2(b)(i) to (v) or a functionally equivalent variant form of a said amino acid sequence.

5 The binding domain may comprise part or all of a VL domain encoded by either a nucleotide sequence as shown in Fig 2(a)(iv) or Fig 2(b)(i) to (v) or a functionally equivalent variant form of a said nucleotide sequence.

10 The binding domain may be specific for both TGFbeta1 and TGFbeta2. The binding domain may be specific for both human TGFbeta1 and human TGFbeta2. The specific binding member may be in the form of scFv.

15 The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 4 or a functionally equivalent variant form of said amino acid sequence. The binding domain may comprise part or all of a VL domain encoded by either the nucleotide sequence as shown in Fig 4 or a functionally equivalent variant form of said nucleotide sequence.

20 The present invention also provides a polypeptide with a binding domain specific for TGFbeta which polypeptide comprises a substantial part or all of either an amino acid sequence as shown in any one of Fig 1(a),Fig 1(b),Fig 1(c),Fig 2(a),Fig 2(b), Fig 4 or a functionally equivalent variant form of a said amino acid sequence. The polypeptide may comprise a substantial part or all of an amino acid sequence which is a functionally equivalent variant form of the Fig 1(a)(i) amino acid sequence, the variant being one of those variants as shown in Fig 3.

25 The present invention also provides a polynucleotide which codes for a polypeptide with a binding domain specific for TGFbeta which polynucleotide comprises a substantial part or all of a nucleotide sequence which codes for either an amino acid sequence as shown in any one of Fig 1(a),Fig 1(b),Fig 1(c),Fig 2(a),Fig 2(b),Fig 4 or a functionally equivalent variant form of a said amino acid sequence. The polynucleotide may code for a polypeptide with a binding domain specific for

TGFbeta which polynucleotide comprises a substantial part or all of a nucleotide sequence which codes for an amino acid sequence which is a functionally equivalent variant form of the Fig 1(a)(i) amino acid sequence, the variant being one of those as shown in Fig 3. The polynucleotide may code for a polypeptide with a binding domain specific for TGFbeta which polynucleotide comprises a substantial part or all of either a nucleotide sequence as shown in any one of Fig 1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig 2(b), Fig 4 or a functionally equivalent variant form of said nucleotide sequence. The polynucleotide may code for a polypeptide with a binding domain specific for TGFbeta which polynucleotide comprises a substantial part or all a nucleotide sequence which codes for a variant form of the Fig 1(a)(i) amino acid sequence, the variant being one of those as shown in Fig 3.

The present invention also provides constructs in the form of plasmids, vectors, transcription or expression cassettes which comprise least one polynucleotide as above.

The present invention also provides a recombinant host cell which comprises one or more constructs as above.

The present invention also provides pharmaceuticals which comprise a specific binding member as above, optionally with one or more excipients.

The present invention also provides the use of a specific binding member as above in the preparation of a medicament to treat a condition in which it is advantageous to counteract the fibrosis promoting effects of TGFbeta. The condition may be a fibrotic condition characterized by an accumulation in a tissue of components of the extracellular matrix. The components of the extracellular matrix may be fibronectin or laminin.

The condition is selected from the group consisting of:

- glomerulonephritis

- neural scarring
- dermal scarring
- lung fibrosis
- arterial injury
- 5 proliferative retinopathy
- retinal detachment
- adult respiratory distress syndrome
- liver cirrhosis
- post myocardial infarction
- 10 post angioplasty restenosis
- keloid scarring
- scleroderma
- vascular disorders
- cataract
- 15 glaucoma.

The condition may be neural scarring or glomerulonephritis.

- 20 The present invention also provides the use of a specific binding member as above, in the preparation of a medicament to treat an immune/inflammatory disease condition in which it is advantageous to counteract the effects of TGFbeta. Illustrative conditions are rheumatoid arthritis, macrophage deficiency disease and macrophage pathogen infection.
- 25 The present invention also provides a method which comprises administering to a patient a therapeutically effective amount of a specific binding member as above in order to treat a condition in which it is advantageous to counteract the fibrosis promoting effects of TGFbeta. Fibrotic conditions are listed above.
- 30 The present invention also provides a method which comprises administering to a patient a prophylactically effective amount of a specific binding member as above in order to prevent a condition in which it is advantageous to prevent the fibrosis

promoting effects of TGF β . Fibrotic conditions are listed above.

The present invention also provides methods which comprise administering to patients prophylactically and/or therapeutically effective amounts of a specific binding member as above in order to prevent or treat an immune/inflammatory disease condition in which it is advantageous to counteract the effects of TGF β . Illustrative conditions are stated above.

The present invention also provides a method which comprises using a construct as stated above, in an expression system in order to express a specific binding member or polypeptide as above.

In order that the present invention is fully understood, the following examples are provided by way of exemplification only and not by way of limitation. Reference is made to the following figures.

Figure 1 shows the DNA and protein sequences of antibodies specific for TGF β 1.

Figure 2 shows the DNA and protein sequences of antibodies specific for TGF β 2.

Figure 3 shows the protein sequences of VH CDR3 of clones derived from 1B2 by 'spiking' mutagenesis.

Figure 4 shows the DNA and protein sequence of the VH and VL domains of VT37.

Figure 5 shows the DNA sequence in the region of the heavy chain VH leader from the vector vh cassette2.

Figure 6 shows a map of the vector pG4D100.

Figure 7 shows the DNA sequence in the region of the light chain VL leader for the vector vlcassette1.

5

Figure 8 shows a map of the vector pLN10.

Figure 9 shows a map of the vector pKN100.

10

Figure 10 shows the neutralisation of TGFbeta2 activity by whole IgG4 antibodies in an assay using proliferation of the erythroleukaemia cell line, TF1.

15

Figure 11 shows the neutralisation of TGFbeta2 activity by single chain Fv antibodies in an assay using proliferation of the erythrolcukaemia cell line, TF1.

20

Figure 12 shows the effect of treatment of animals with antibodies on neural scarring as measured by the deposition of (a) fibronectin and (b) laminin detected using integrated fluorescence intensity. The graphs show scatter plots of individual animal data points. The bar graph shows the mean of the group.

25

Figure 13 shows the results of an ELISA to measure the cross-reactivity of the antibodies 6B1 IgG4 and 6A5 IgG4 with TGFbeta isoforms and non-specific antigens.

25

Figure 14 shows the amount of urinary protein in 24h measured for rat groups A to E in the experimental glomerulonephritis model.

30

Figure 15 shows the periodic acid Schiff matrix score (derived by measurement of the amount of staining) for rat groups A to E in the experimental glomerulonephritis model.

List of Examples

Example 1 - Isolation of antibodies specific for TGFbeta1, antibodies specific for TGFbeta2 and antibodies specific for TGFbeta1 and TGFbeta2.

Example 2 - Construction of cell lines expressing whole antibodies.

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Example 3 - Neutralisation of TGFbeta activity by antibodies assessed using in vitro assays.

Example 4 - Inhibition by antibodies of TGFbeta binding to receptors

10.

Example 5 - Prevention of neural scarring using antibodies against TGF β

Example 6 - Prevention of glomerulonephritis using antibodies against TGF β

Example 1 Isolation and characterisation of antibodies binding to TGF β 1 and TGF β 2

5 1. Identification and Characterisation of Antibodies to Human TGF β -1 by Selection of Naive and Synthetic Phage Antibody Repertoires

10 Antibody repertoires

The following antibody repertoires were used:

15 1. Peripheral blood lymphocyte (PBL) library derived from unimmunized human (Marks, J. D., Hoogenboom, H. R. Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991) J. Mol. Biol. 222, 581-597)

20 2. Synthetic library (Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994) EMBO J. 13, 692-698) derived from cloned human germline VH genes and synthetic CDR3s with a fixed light chain

25 3. Tonsil library derived from the tonsils of unimmunised humans. Tonsil B cells were isolated from freshly removed (processed within 2 hours) whole tonsils provided by Addenbrookes Hospital, Hills Road, Cambridge, U.K. Each tonsil was processed as follows. Tonsils were placed in a petri dish containing 5ml of PBS and macerated with a scalpel blade to release the cells. The suspension was transferred to a fresh tube and large debris allowed to sediment under gravity for 5 minutes. The cell suspension was then overlaid onto 10mls of Lymphoprep in a 50 ml polypropylene tube (Falcon) and centrifuged at 1000xg 20 minutes at room temperature (no brake) and cells at the interface harvested with a glass pipette. These were diluted to a final volume of 50 ml in RPMI medium at 37° C and centrifuged at 500xg for 15 minutes at room temperature. The supernatant was aspirated and the the cells washed another two times with RPMI.

30 Polyadenylated RNA was prepared from pelleted cells using the

5 "Quickprep™ mRNA Kit" (Pharmacia Biotech, Milton Keynes, U.K.). The entire output of cells from one tonsil (ca. 1×10^6 cells) was processed using one Oligo(dT)-Cellulose Spun column and processed exactly as described in the accompanying protocol. mRNA was ethanol precipitated as described and resuspended in 40ml RNase free water.

10 The cDNA synthesis reaction was set up using the "First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Milton Keynes, U.K.) as follows:

RNA-	20 μ l (heated to 67 °C 10 minutes before use)
1st strand buffer-	11 μ l
15 DTT solution	1 μ l
pd(N) ₆ primer	1 μ l

20 After gentle mixing, the reaction was incubated at 37 °C for 1 hour.

25 Human VH genes were amplified from tonsil cDNA using the nine family-based back primers (VH 1b/7a -6a back *Sfi* I, which introduce a *Sfi* I site at the 5'-end, Table 1) together with an equimolar mixture of the four JH forward primers (JH 1-2, 3, 4-5, 6. for; Marks et al., 1991 supra). Thus, nine primary PCR amplifications were performed. Each reaction mixture (50 μ l) comprised 2 μ l cDNA template, 25 pmol back primer, 25 pmol forward primers, 250 μ M dNTPs, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 2.5 u of *Taq* polymerase (Boehringer). The reaction mixture was overlaid with mineral (paraffin) oil and was cycled 30 times (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) using a Techne thermal cycler. The products were purified on a 1% (w/v) agarose gel, isolated from the gel using "GeneClean" (Bio 101 Inc.) and resuspended in 15 μ l of water. The amplified VH genes were recombined with human VL genes derived from PB1.s (Marks et al., 1991 supra) together with the (Gly₄, Ser)₃ linker (Fluton, J.S., et al. 1988 Proc Natl Acad Sci U S A. 85: 5879-83) by PCR assembly (Marks et al, 1991 supra). The VH-linker-VL antibody constructs were cloned into the *Sfi*I and *Not*I sites of the phagemid vector, pCANTAB6 (McCafferty, J., et al. 1994 Appl. Biochem. Biotech. 47: 157 - 173) to give a library of 6×10^7 clones.

4. Large single chain Fv library derived from lymphoid tissues including tonsil, bone marrow and peripheral blood lymphocytes.

Polyadenylated RNA was prepared from the B-cells of various lymphoid tissues of 43
5 non-immunised donors using the "Quickprep mRNA Kit" (Pharmacia). First-strand
cDNA was synthesized from mRNA using a "First-strand cDNA synthesis" kit
(Pharmacia) using random hexamers to prime synthesis. V-genes were amplified
using family-specific primers for VH, V κ and V λ genes as previously described
(Marks et al., supra) and subsequently recombined together with the (Gly₄, Ser)₃ scFv
linker by PCR assembly. The VH-linker-VL antibody constructs were cloned into the
Sfi I and Not I sites of the phagemid vector, pCANTAB 6. Ligation, electroporation
and plating out of the cells was as described previously (Marks et al, 1991 supra). The
library was made ca. 1000x larger than that described previously by bulking up the
amounts of vector and insert used and by performing multiple electroporations. This
10 generated a scFv repertoire that was calculated to have ca. 1.3×10^{10} individual
recombinants which by *Bst* NI fingerprinting were shown to be extremely diverse.
15

a. Induction of phage antibody libraries

20

The four different phage antibody repertoires above were selected for antibodies to
TGF β -1. The VH synthetic (Nissim et al., 1994 supra), tonsil, 'large' scFv and PBL
(Marks et al., 1991 supra) repertoires were each treated as follows in order to rescue
phagemid particles. 500 ml prewarmed (37 °C) 2YTAG (2YT media supplemented
25 with 100 μ g/ml ampicillin and 2 % glucose) in a 2 l conical flask was inoculated with
approximately 3×10^{10} cells from a glycerol stock (-70 °C) culture of the appropriate
library. The culture was grown at 37 °C with good aeration until the OD_{600nm} reached
0.7 (approximately 2 hours). M13K07 helper phage (Stratagene) was added to the
culture to a multiplicity of infection (moi) of approximately 10 (assuming that an
30 OD_{600nm} of 1 is equivalent to 5×10^8 cells per ml of culture). The culture was
incubated stationary at 37 °C for 15 minutes followed by 45 minutes with light
aeration (200 rpm) at the same temperature. The culture was centrifuged and the

supernatant drained from the cell pellet. The cells were resuspended in 500 ml 2YTAK (2YT media supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin), and the culture incubated overnight at 30 °C with good aeration (300 rpm). Phage particles were purified and concentrated by three polyethylene glycol (PEG) precipitations (Sambrook, J., Fritsch, E.F., & Maniatis, T. (1990). Molecular Cloning - A Laboratory Manual. Cold Spring Harbour, New York) and resuspended in PBS to 10¹² transducing units (tu)/ml (ampicillin resistant clones).

10 **b. Panning of phage antibody library on TGFβ-1**

Phage induced from the four repertoires were each separately panned on TGFβ-1. A 75mm x 12mm immuno tube (Nunc; Maxisorp) was coated with 2 ml of recombinant human TGFβ-1 (0.5µg/ml, Genzyme) in PBS overnight at 4 °C. After washing 3 times with PBS, the tube was filled with 3%MPBS (3 % 'Marvel' skimmed milk powder, 1x PBS) and incubated for 2 hours at 37 °C for blocking. The wash was repeated, phagemid particles (10¹³ tu) in 2 ml of 3% MPBS were added and the tube incubated stationary at 37 °C for 1 hour. The tube was washed 20 times with PBST(0.1%), then 20 times with PBS. Bound phage particles were eluted from the tube by adding 2 ml of 100mM-triethylamine, and incubating the tube stationary at room temperature for 10 minutes. The eluted material was immediately neutralised by pipetting into a tube containing 1 ml 1M-Tris.HCl (pH7.4). Phage were stored at 4 °C. 1.5 ml of the eluted phage were used to infect 20 ml of logarithmically growing E. coli TG1 (Gibson, T.J. (1984). PhD thesis. University of Cambridge, UK.).

15 Infected cells were grown for 1 hour at 37 °C with light aeration in 2YT broth, and then plated on 2YTAC medium in 243mm x 243mm dishes (Nunc). Plates were incubated overnight at 30 °C. Colonies were scraped off the plates into 10 ml of 2YT broth and 15 % (v/v) glycerol added for storage at -70 °C.

20 Glycerol stock cultures from the first round of panning of each of the four repertoires on TGFβ-1 were each rescued using helper phage to derive phagemid particles for the second round of panning. 250 µl of glycerol stock was used to inoculate 50 ml

2YTAK broth, and incubated in a 250 mL conical flask at 37 °C with good aeration until the OD_{600nm} reached 0.7 (approximately 2 hours). M13K07 helper phage (moi=10) was added to the culture which was then incubated stationary at 37 °C for 15 minutes followed by 45 minutes with light aeration (200 rpm) at the same temperature. The culture was centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 50 ml prewarmed 2YTAK, and the culture incubated overnight at 30 °C with good aeration. Phage particles were purified and concentrated by PEG precipitation (Sambrook et al., 1990 *supra*) and resuspended in PBS to 1013 tu/ml.

10

Phage induced from the first round of panning of each of the three repertoires, was selected a second time essentially as described above except that the panning tube was coated with only 1 ml of TGFβ-1 (0.5ug/ml, Genzyme), and the volume of phage added to the tube similarly reduced. After extensive washing, bound phage were eluted from the tube using 1 ml of 100 mM-triethylamine, and neutralised by the addition of 0.5 ml 1M-Tris.HCl (pH7.4) as earlier described. The process of phage growth and panning was repeated over a third and a fourth round of selection.

20

c. Growth of single selected clones for immunoassay

25

Individual colonies from the third and fourth round selections were used to inoculate 100 µl 2YTAK into individual wells of 96 well tissue culture plates (Corning). Plates were incubated at 30 °C overnight with moderate shaking (200 rpm). Glycerol to 15 % was added to each well and these master plates stored at -70 °C until ready for analysis.

30

d. ELISA to identify anti-TGFβ-1 scFv

Clones specific for TGFβ-1 were identified by ELISA, using scFv displayed on phage or soluble scFv.

i. Phage ELISA

Cells from the master plates were used to inoculate fresh 96 well tissue culture plates containing 100 µl 2YTAK per well. These plates were incubated at 37 °C for 6-8 hours or until the cells in the wells were growing logarithmically (OD600 0.2-1.0). M13K07 was added to each well to an moi of 10 and incubated stationary for 15 min then 45 min with gentle shaking (100 rpm), both at 37 °C. The plates were centrifuged at 2000 rpm for 10 min and the supernatant eluted. Each cell pellet was resuspended in 100 µl 2YTAK and incubated at 30 °C overnight.

Each plate was centrifuged at 2000 rpm and the 100 µl supernatant from each well recovered and blocked in 20 µl 18%M6PBS (18 % skimmed milk powder, 6 x PBS), stationary at room temperature for 1 hour. Meanwhile, flexible microtitre plates which had been blocked overnight stationary at 4 °C with either 50 µl 0.2 µg/ml TGF β -1 in PBS or 50 µl PBS alone (giving an uncoated control plate), were washed 3 times in PBS and blocked for 2 h stationary at 37 °C in 3MPBS. These plates were then washed three times with PBS and 50 µl preblocked phage added to each well of both the TGF β -1-coated or uncoated plate. The plates were incubated stationary at 37 °C for 1 h after which the phage were poured off. The plates were washed by incubating for 2 min in PBST three times followed by incubating for 2min in PBS three times, all at room temperature.

To each well of both the TGF β -1-coated and the uncoated plate, 50 µl of a 1 in 10,000 dilution of sheep anti-fd antibody (Pharmacia) in 3MPBS was added and the plates incubated at 37 °C stationary for 1 h. Each plate was washed as described above and 50 µl of a 1 in 5,000 dilution donkey anti-sheep alkaline phosphatase conjugate (Sigma) in 3MPBS added and incubated stationary at 37 °C for 1 h. Plates were washed as described as above followed by two rinses in 0.9% NaCl. Alkaline phosphatase activity was visualised using either the chromogenic substrate pNPP (Sigma) or the Ampak system (Dako). The absorbance signal generated by each clone was assessed by measuring the optical density at either 405 nm (pNPP) or 492

nm (Ampak) using a microtitre plate reader. Clones were chosen for further analysis if the ELISA signal generated on the TGF β -1-coated plate was at least double that on the uncoated plate.

5

ii. Soluble ELISA

Cells from the master plates were used to inoculate fresh 96 well tissue culture plates containing 100 μ l 2YTAG per well. These plates were incubated at 30 °C for 8 hours then centrifuged at 2000 rpm for 10 min and the supernatant eluted. Each cell pellet was resuspended in 100 μ l 2YTA (2YT media supplemented with 100 μ g/ml ampicillin) containing 10 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and incubated at 30 °C overnight.

15 Each plate was centrifuged at 2000 rpm and the 100 μ l supernatant from each well recovered and blocked in 20 μ l 18%MPBS stationary at room temperature for 1 hour. Meanwhile, flexible microtitre plates which had been blocked overnight stationary at 4 °C with either 50 μ l 0.2 μ g/ml TGF β -1 in PBS or 50 μ l PBS alone, were washed 3 times in PBS and blocked for 2 h stationary at 37 °C in 3%MPBS.

20 These plates were then washed three times with PBS and 50 μ l preblocked soluble scFv added to each well of both the TGF β -1-coated or uncoated plate. The plates were incubated stationary at 37 °C for 1 h after which the scFv solutions were poured off. The plates were washed by incubating for 2 min in PBST (PBS containing 1% Tween) three times followed by incubating for 2 min in PBS three times, all at room 25 temperature.

To each well of both the TGF β -1-coated and the uncoated plate, 50 μ l of a 1 in 200 dilution of the anti-myc tag murine antibody 9E10 (Munro, S. & Pelham, H.R.B. (1986)Cell 46, 291-300) in 3MPBS was added and the plates incubated at 37 °C 30 stationary for 1 h. Each plate was washed as described above and 50 μ l of a 1 in 5,000 dilution goat anti-mouse alkaline phosphatase conjugate (Pierce) in 3MPBS added and incubated stationary at 37 °C for 1 h. Plates were washed as described

above followed by two rinses in 0.9% NaCl. Alkaline phosphatase activity was visualised using either the chromogenic substrate pNPP (Sigma) or the Ampak system (Dako). The absorbance signal generated by each clone was assessed by measuring the optical density at either 405 nm (pNPP) or 492 nm (Ampak) using a microtitre plate reader. Clones were chosen for further analysis if the ELISA signal generated on the TGF β -1-coated plate was at least double that on the uncoated plate.

5

iii. Specificity ELISA

10

Clones identified as binding TGF β -1 rather than uncoated well, as described above, were further analysed for fine specificity. Specificity ELISA's were carried out using scFv either displayed on phage or in solution as described above, except that 5 ml of media in 50 ml Falcon tubes were inoculated with each clone and grown to generate the phage or soluble scFv used in the ELISA. Microtitre plate wells were coated with 50 μ l of either 0.2 μ g/ml TGF β -1, 0.2 μ g/ml TGF β -2, 10 μ g/ml bovine serum albumin (BSA) or PBS (the uncoated well). After preblocking both the phage (or soluble scFv) and the microtitre plates, 50 μ l blocked phage (or soluble scFv) from each clone was added to a well coated with either TGF β -1, TGF β -2, BSA or an uncoated well. As above, alkaline phosphatase activity was visualised using either the chromogenic substrate pNPP (Sigma) or the Ampak system (Dako). Clones were considered to be specific for TGF β -1 if the ELISA signal generated in the TGF β -1 coated well was at least five-fold greater than the signal on either TGF β -2, BSA or an uncoated well.

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20

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iv. Specificity determination by BIACore

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The antibodies were also shown to be specific for TGF β 1 compared to TGF β 2 (obtained from R&D Systems Abingdon) by relative binding to the BIACore sensor chips coated with the appropriate antigen. TGF β 1 and TGF β 2 were immobilised by amine coupling to Biosensor CM5 sensorchips (Pharmacia) according to the manufacturers instructions. Single chain Fv fragments (35 μ l; purified by immobilized

metal affinity chromatography as described in example 4) were injected over the immobilized antigen at a flow rate of 5 μ l/min. The amount of TGF β bound was assessed as the total increase in resonance units (RUs) over this period. For 31G9 scFv an increase of 1059RUs was found with a TGF β 1 chip and 72 RUs was found with a TGF β 2 chip. Thus binding is much stronger to TGF β 1 than TGF β 2.

5

e. Sequencing of TGF β 1-Specific ScFv Antibodies

The nucleotide sequence of the TGF β -1 specific antibodies was determined by first using vector-specific primers to amplify the inserted DNA from each clone. Cells from an individual colony on a 2YTAG agar plate were used as the template for a polymerase chain reaction (PCR) amplification of the inserted DNA using the primers pUC19reverse and fdtetseq (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by 10 min at 10 72 °C. The PCR products were purified using a PCR Clean-up Kit (Promega) in to a final volume of 50 μ l H₂O. Between 2 and 5 μ l of each insert preparation was used as the template for sequencing using the Taq Dye-terminator cycle sequencing system (Applied Biosystems). The primers mycseq10 and PCR-L-Link were used to sequence the light chain of each clone and PCR-H-Link and pUC19reverse to sequence the heavy chain (Table 1)

15

20

f. Sequence and Source of the Initial TGF β -1-Specific ScFv Antibodies

25

Four different TGF β -1 specific antibodies were isolated from the selections using the four libraries described above. Each clone name, its origin and its heavy and light chain germline is given below. The complete sequence of each VH domain gene is given in figure 1(a), together with the VL domain gene, from scFv 31G9.

30

CLONE	LIBRARY SOURCE	VH GERMLINE	VL ISOTYPE
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	1-B2	PBL	VH3	DP49	VKappa
	IA-E5	Synthetic VH	VH3	DP53	VLambda
5	1A-H6	Tonsil	VH3	DP50	VLambda
	31-G9	large scFv	VH3	DP49	VLambda

Thus these initial isolates were obtained from libraries derived from different sources-both natural V genes of unimmunised humans and synthetic libraries from cloned germline V genes together with synthetic CDRs.

2. Affinity Maturation of the Initial TGF β -1-Specific ScFv Antibodies

a. Light Chain Shuffling of the TGF β -1-Specific ScFv Antibody 1-B2

i. Construction of Repertoires

The heavy chain of clone 1-B2 was recombined with the complete repertoire of light chains derived from the PBL and large (tonsil-derived) scFv repertoires. The 1-B2 heavy chain was amplified by PCR using the primers HuJh4-5For (Table 1) and pUC19reverse. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

The PBL and tonsil light chains were amplified by PCR using the primers fdtetseq and a mix of RL1, 2 & 3 (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VL excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

Approximately 50 ng amplified 1-B2 heavy chain and 50 ng of either

amplified PBL-derived or amplified tonsil-derived light chains were combined and precipitated with sodium acetate and ethanol using 25 µg glycogen as a carrier. The precipitated DNA was pelleted by centrifugation at 13,000 rpm in a microfuge, air dried and resuspended in 26 µl H₂O. This was used in an assembly amplification after
5 the addition of reaction buffer to 1X, dNTP's to 200 nM and 5 units Taq polymerase. Amplification conditions consisted of 20 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1min 30 s, followed by 10 min at
10 72 °C. 10 µl of each assembly was used as the template in a 'pull-through' amplification with the primers fdtetseq and pUC19reverse. Amplification conditions consisted of 25 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1min 30 s,
followed by 10 min at 72 °C.

The pull-through amplification product was separated through 1% agarose-TAE and the band representing the pull-through VH-VL excised and eluted using the Geneclean Kit. This was digested with the restriction endonucleases Sfi I
15 and Not I (NEB) and ligated (Amersham ligation system) into the phagemid vector pCantab 6, previously digested with Sfi I and Not I. The ligation product was used to transform electrocompetent TG1 cells, plated out on 2YTAG plates and incubated overnight at 30 °C. Approximately 1 x 10³ individual clones were generated from the light chain-shuffle of the 1-B2 heavy chain with the PBL-derived light chains and
20 approximately 1 x 10⁶ for the shuffle with the tonsil-derived light chains.

ii. Selection of Light Chain Shuffle Repertoires

25 The two light chain-shuffle repertoires were selected for TGFβ-1-specific antibodies. Phagemid particles were recovered from each repertoire as described earlier for the initial libraries. Recovered phage were preblocked for 1 h in a final volume of 100 µl 3MPBS. Approximately 10¹¹ tu phage were used in the first round selection and between 10⁹ and 10¹⁰ for subsequent selections. For the first round selections,
30 biotinylated TGFβ1 to a final concentration of 100 nM was added to the preblocked phage and incubated stationary at 37°C for 1h.

For each selection, 100 μ l Dynabeads suspension (Dynal) was separated on a magnet and the beads recovered and preblocked for 2 h in 1 ml 3MPBS. The beads were recovered on a magnet and resuspended in the phagemid/biotinylated TGF β -1 mixture and incubated at room temperature for 15 min while being turned end-over-end. The beads were captured on a magnet and washed four times with PBST followed by three washes in PBS. After each wash, the beads were captured on a magnet and resuspended in the next wash. Finally, half of the beads were resuspended in 10 μ l 50 mM DTT (the other half of the beads stored at 4 °C as a back-up) and incubated at room temperature for 5 min. The whole bead suspension was then used to infect 5 ml logarithmically-growing TG1 cells. This was incubated at 37 °C, stationary for 15 min then with moderate shaking for 45 min, plated on 2YTAG plates and incubated overnight at 30 °C.

Colonies were scraped off the plates into 10 ml of 2YT broth and 15 % (v/v) glycerol added for storage at -70 °C. A 250 μ l aliquot of each plate scrape was used to inoculate 2YTAG and phagemid particles rescued as described earlier. For each repertoire, three rounds of selection using biotinylated TGF β -1 was performed, essentially identical to the first round selection described above. All selections were at 100 nM TGF β -1 except for the third round selection of the tonsil-derived light chain repertoire where the concentration of biotinylated TGF β -1 in the selection was reduced to 50 nM.

iii. Identification of TGF β -1-Specific ScFv Antibodies from Light Chain Shuffle
25 Repertoires

ScFv antibodies specific to TGF β -1 were identified by both phage and soluble ELISA, and sequenced, as described earlier. Three new TGF β -1-specific scFv antibodies were identified, two with PBL-derived light chains and one with a tonsil-derived light chain. All three had the 1B2 heavy chain sequence (DP49), described earlier. The sequences are summarised below and the complete sequence of each VL domain gene is given in figure 1(b).

CLONE	VL SOURCE	VH GERMLINE	VL ISOTYPE
7-A3	PBL	DP49 (1B2)	VKappa
10-A6	PBL	DP49 (1B2)	VLambda
14-A1	Tonsil	DP49 (1B2)	VLambda

Thus the VH domain 1B2 derived from the PBL library can be combined with VL domains derived from both PBL and tonsil libraries.

b. CDR3 'Spiking' of the TGF β -1-Specific ScFv Antibody 1B2

15

i. Construction of 'spiked' repertoire

An 84 mer mutagenic oligonucleotide primer, 1B2 mutVHCDR3, was first synthesized (see Table 1). This primer was 'spiked' at 10%; i.e. at each nucleotide position there is a 10% probability that a non-parental nucleotide will be incorporated. The 1-B2 heavy chain was amplified by PCR using the primers pUC19reverse and 1B2 mutVHCDR3. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

The parental 1B2 light chain was amplified by PCR using the primers fdtetseq and RL3 (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VL excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

101).

Approximately 50 ng amplified 'spiked' 1-B2 heavy chain and 50 ng of amplified parental 1B2 light chain were combined and precipitated with sodium acetate and ethanol using 25 µg glycogen as a carrier. The precipitated DNA was pelleted by centrifugation at 13,000 rpm in a microfuge, air dried and resuspended in 26 µl H₂O. This was used in an assembly amplification after the addition of reaction buffer to 1X, dNTP's to 200 nM and 5 units Taq polymerase. Amplification conditions consisted of 25 cycles of 94 °C for 1 min, 65 °C for 4 min. Five µl of each assembly was used as the template in a 'pull-through' amplification with the primers fdtetseq and pUC19reverse. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1 min, followed by 10 min at 72 °C.

The pull-through amplification product was separated through 1% agarose-TAE and the band representing the pull-through 'spiked' VH - VL excised and eluted using the Geneclean Kit. This was digested with the restriction endonucleases Sfi I and Not I (NEB) and ligated (Amersham ligation system) into the phagemid vector pCantab 6, previously digested with Sfi I and Not I. The ligation product was used to transform electrocompetent TG1 cells, plated out on 2YTG plates and incubated overnight at 30 °C. Approximately 4 x 10⁶ individual clones were generated from this VH CDR3 'spiking' of the 1-B2 VH CDR3.

20

ii. Selection of 1B2 CDR3 Spike Repertoire

The repertoire was selected for new TGFβ-1-specific scFv antibody by one round of panning on 1 µg/ml TGFβ-1 followed by two rounds of selection with biotinylated TGFβ-1 at 50 nM using methods as described earlier.

30 iii. Identification of TGFβ-1-Specific ScFv Antibodies from the 1B2 CDR3 Spike Repertoire

ScFv antibodies specific to TGFβ-1 were identified by both phage and soluble and

phage ELISA, and sequenced, as described earlier. Clone 27C1 was isolated from the spiked repertoire. It is virtually identical to clone 1B2 but with three differences in the heavy chain CDR3. The complete sequence of clone 27C1 is given in figure 1 (c). The 27C1 VH domain was combined with the 10A6 VL domain in the construction of the whole antibody 27C1/10A6 IgG4 (example 2). The properties of this antibody are described in more detail in examples 2 to 6. In addition to 27C1, a large number of other antibodies were isolated with up to 7 of the 14 amino acids differing in CDR3 of the VH domain (Figure 3). These had a similar preference for binding TGFbeta1 compared to TGFbeta2.

3. Identification and Characterisation of Antibodies to Human TGF β -2 by Selection of Naive and Synthetic Phage Antibody Repertoires

5

a. Induction of phage antibody libraries

Two different phage antibody repertoires were selected for antibodies to TGF β -2. The VH synthetic (Nissim et al., 1994) and tonsil (constructed as described earlier) repertoires were each treated as described for TGF β -1 to rescue phagemid particles.

10

b. Panning of phage antibody library on TGF β -2

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Phage induced from the two repertoires were each separately panned on TGF β -2 as described earlier for TGF β -1 but using 0.5 μ g/ml TGF β -2 as the coating antigen.

20

Individual colonies from the third and fourth round selections were screened by both phage and soluble ELISA as described earlier for TGF β -1 but using flexible microtitre plates coated with TGF β -2 at 0.2 μ g/ml rather than TGF β -1. Clones were chosen for further analysis if the ELISA signal generated on the TGF β -2-coated plate was at least double that on the uncoated plate. For the specificity ELISA, as described earlier for TGF β -1, clones were considered to be specific for TGF β -2 if the ELISA signal generated in the TGF β -2 coated well was at least five-fold greater than the signal on either TGF β -1, BSA or an uncoated well.

25

c. Identification and Sequencing of TGF β -2-Specific ScFv Antibodies

30

Four different TGF β -2 specific antibodies were isolated from the selections using the two libraries described above. Each clone name, its origin and its heavy and light

chain germline is given below. The complete sequence of each VH domain gene is given in figure 2 (a) together with the VL domain of Gold-11.

	CLONE	LIBRARY SOURCE	VH GERMLINE	VL ISOTYPE
5	1-G2	Tonsil		
10	1-H6	Tonsil	DP49	
	2A-H11	Synthetic VH	DP50	VLambda
	2A-A9	Synthetic	DP46	VLambda
15	Gold-11	Large scFv		VLambda

Thus human antibodies binding to human TGFbeta2 have been isolated from different sources-, both natural V genes of unimmunised humans and synthetic libraries from cloned germline V genes together with synthetic CDRs.

20

4. Light Chain Shuffling of the TGF β -2-Specific scFv Antibodies 2A-H11 and 2A-A9

5

a. Construction of Repertoires

The heavy chain of clones 2A-H11 and 2A-A9 were recombined with the complete repertoire of light chains derived from the PBL and large (tonsil-derived) scFv repertoires as described earlier for the TGF β -1-specific scFv antibody 1-B2. Both repertoires generated from the recombination with the PBL light chain repertoire were approximately 1×10^5 , those generated from the recombination with the tonsil light chain repertoire were approximately 1×10^6 .

15

b. Selection of Light Chain Shuffle Repertoires

The light chain-shuffle repertoires were selected for TGF β -2-specific antibodies using biotinylated TGF β -2, as described earlier for the selection of the TGF β -1 light chain shuffle repertoires. For all of the first and second round selections, a concentration of 100 nM biotinylated TGF β -2 was used. For the third round selection of the PBL-derived light chain shuffle repertoire, biotinylated TGF β -2 was used at concentrations of 100 nM and 1 nM. For the third round selection of the tonsil-derived light chain shuffle repertoire, biotinylated TGF β -2 was used at a concentration of 50 nM.

c. Identification of TGF β -2-Specific scFv Antibodies from Light Chain Shuffle Repertoires

ScFv antibodies specific to TGF β -2 were identified by both phage and soluble ELISA, and sequenced, as described earlier. Five new TGF β -2-specific scFv antibodies were identified. The sequences are summarised below and the complete

sequence of each clone given in figure 2 (b).

	CLONE	VL SOURCE	VH GERMLINE	VL ISOTYPE
5	6-H1	PBL	DP50 (2A-H11)	VKappa
	6-A5	PBL	DP50 (2A-H11)	VLambda
10	6-B1	PBL	DP50 (2A-H11)	VLambda
	11-E6	PBL	DP46 (2A-A9)	VKappa
	14-F12	Tonsil	DP46 (2A-A9)	VLambda

15 **d. Specificity determination by ELISA**

Clones identified as binding TGF β -2 rather than uncoated well, as described above, were further analysed for fine specificity. Specificity ELISA's were carried out using scFv either displayed on phage or in solution as described above, except that 5 ml of media in 50 ml Falcon tubes were inoculated with each clone and grown to generate the phage or soluble scFv used in the ELISA. Microtitre plate wells were coated with 50 μ l of either 0.2 μ g/ml TGF β -1, 0.2 μ g/ml TGF β -2, 10 μ g/ml bovine serum albumin (BSA) or PBS (the uncoated well). After preblocking both the phage (or soluble scFv) and the microtitre plates, 50 μ l blocked phage (or soluble scFv) from each clone was added to a well coated with either TGF β -1, TGF β -2, BSA or an uncoated well. As above, alkaline phosphatase activity was visualised using either the chromagenic substrate pNPP (Sigma) or the Ampak system (Dako). Clones were considered to be specific for TGF β -2 if the ELISA signal generated in the TGF β -2 coated well was at least five-fold greater than the signal on either TGF β -2, BSA or an uncoated well. Cross-reactivity with unrelated antigens was determined more extensively for anti-TGF β 2 antibody in whole antibody format, see example 2. The cross-reactivity of 6B1 IgG4 and 6A5 IgG4 with TGF β 1 and TGF β 3 (obtained

from R&D Systems, Abingdon) is also shown to be very low.

c. Specificity determination by BIACore

5 The antibodies were also shown to be specific for TGFbeta2 compared to TGFbeta1 by relative binding to the BIACore sensor chips coated with the appropriate antigen. TGFbeta1 and TGFbeta2 were immobilised by amine coupling to Biosensor CMS sensorchips (Pharmacia) according to the manufacturers instructions. Single chain Fv fragments (35µl; purified by immobilized metal affinity chromatography) were
10 injected over the immobilized antigen at a flow rate of 5µl/min. The amount of TGFbeta bound was assessed as the total increase in resonance units (RUs) over this period. For the single chain Fv fragments 6H1, 6A5 and 14F12, these fragments gave a total of 686, 480 and 616 RUs respectively for the TGFbeta1 coated sensor chip and 77, 71 and 115 RUs respectively for the TGFbeta2 coated chip.

15

5. Building higher affinity anti TGF β -1 biological neutralisers**a. Recombining heavy chains derived from high affinity anti- TGF β 1 scFv with light chains derived from anti -TGF β 1 and anti-TGF β 2 scFv showing good properties**

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10

Antibodies derived by spiking CDR3 of the scFv antibody 1-B2 (section 2b) bind TGF β -1 with high affinity. To improve the chance of obtaining high affinity neutralising antibodies it was decided to chain shuffle VHs derived from high affinity anti-TGF β -1 scFv with VLs derived from scFv clones with promising properties and particularly with those capable of neutralising the activity of TGF β -2 *in vitro*.

15

Heavy chains were amplified by PCR from the repertoire of CDR3 spiked 1-B2 clones after selection on TGF β -1 (section 2a.ii) using the primers pUC19reverse and PCR-H-Link (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

20

25

Light chains were separately amplified by PCR from each of the anti TGF β -1 specific neutralisers (7-A3, 10-A6 and 14-A1; section 2a.iii) and each of the anti TGF β -2 specific neutralisers (6H11, 6A5, 6B1, 11E6 and 14F12; section 4c) using the primers fdtetseq1 and PCR-L-Link (Table 1). The same PCR conditions were used as described for the VH amplification. Each VL PCR product was then separately purified through a 1% agarose-TAE gel as described above. Purified products were finally mixed in approximately equimolar amounts (as estimated from an analytical agarose gel) to provide a VL 'pool'.

30

Approximately 50 ng amplified heavy chains and 50 ng of amplified pooled light chains were combined and precipitated with sodium acetate and ethanol using 25 μ g glycogen as a carrier. The precipitated DNA was pelleted by centrifugation at 13,000

rpm in a microfuge, air dried and resuspended in 23 µl H₂O. This was used in an assembly amplification after the addition of reaction buffer, dNTP's to 200 nM and 5 units Taq polymerase. Amplification conditions consisted of 20 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 mins, followed by 10 min at 72 °C. 5 µl of assembly was used as the template in a 50ul 'pull-through' amplification with the primers fdtetseq and pUC19reverse. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2mins, followed by 10 min at 72 °C.

The pull-through amplification product was separated through 1% agarose-TAE and the band representing the pull-through VII-VL excised and eluted using the Geneclean Kit. This was digested with the restriction endonucleases Sfi I and Not I (NEB) and ligated into the phagemid vector pCantah 6 (McCafferty et al. 1994 supra), previously digested with Sfi I and Not I, using the Amersham ligation system. The ligation product was used to transform electrocompetent TG1 cells, plated out on 2YTAG plates and incubated overnight at 30 °C. A repertoire of approximately 3 x 10⁶ individual clones was generated.

b. Selection of chain shuffled repertoire

The chain shuffled repertoire was selected by a single round of panning on TGFβ-1 (1ug/ml), as previously described (section 1b).

c. Identification of TGFβ-1 specific scFv antibodies

ScFv antibodies specific to TGFβ-1 were identified by phage ELISA and sequenced as described earlier (sections 1d.i and 1e). New TGFβ-1 specific scFv antibodies were identified. Two new high affinity clones were isolated -CS32 which consists of 31G9 VII and 7A3 VL and CS39 which consists of 31G9 VII and 6H1 VL.

d. Off-rate determination for single chain Fv fragments binding to TGFbeta1 and TGFbeta2

The off-rates for binding to TGF β 1 or TGF β 2 of the single chain Fv fragments described in this example were determined as described by Karlsson et al (R. Karlsson et al, J. Immunol. Methods 145, 229-240, 1991). The results obtained are shown in Table 2, together with dissociation constants for those which have been determined. These results indicate that high affinity antibodies have been isolated.

5

6. Identification and Characterisation of an Antibody which Cross-reacts with both Human TGF β -1 and TGF β -2 but not TGF β -3 by Selection of a Large scFv Repertoire

10

a. Panning of the Library and Identification of Binders

15

The large scFv library (described earlier) was induced, phagemid particles rescued and panned as described earlier with the following modifications. For the first round of panning, 1012 tu library phage in 0.5 ml PBS were used (rather than the standard 2 ml), for the second round, 3.5×10^9 phage in 0.5 ml PBS were used. The immunotube was coated with 10 μ g TGF β -2 in 0.5 ml PBS for both the first and second round of selection. Individual colonies from the second selection were screened by ELISA using 0.2 μ g/ml TGF β -1. Clones binding TGF β -1 were further screened on TGF β -2, TGF β -3, BSA and PBS. Clones were considered to be specific for both TGF β -1 and TGF β -2 if the ELISA signal generated in the TGF β -1 and the TGF β -2 coated wells were both at least five-fold greater than the signal on TGF β -3, BSA and an uncoated well.

20

25

c. Identification of a TGF β -1/TGF β -2 Cross-reactive scFv Antibody

30

A single scFv antibody specific for both TGF β -1 and TGF β -2 was identified by both phage and soluble ELISA, and sequenced, as described earlier. The complete sequence of the VL domain of the antibody gene VT37 is given in figure 4. The

dissociation constant of this single chain Fv antibody was estimated by analysis using BIACore to be 4nM for TGFbeta1 and 7nM for TGFbeta2. Cross-reactivity for TGFbeta3 was also determined. Purified VT37scFv at 8.3 μ g/ml was passed over BIACore sensor chips coated with TGFbeta1 (500RUs coated); TGFbeta2 (450RUs coated) or TGFbeta3 (5500RUs coated). The relative response for VT37 scFv binding was: TGFbeta1 - 391RU bound; TGFbeta2 - 261RU bound or TGFbeta3 - 24RU bound. Thus this antibody binds strongly to TGFbeta1 and TGFbeta2 but binding to TGF beta 3 is not detectable above background.

10 **Example 2 Construction of Cell Lines Expressing Whole Antibodies**

For the construction of cell lines expressing IgG4 antibodies, variable domains were cloned into vectors expressing the human gamma 4 constant region for the VH domains or the human kappa or lambda constant regions for the VL domains.

15 To construct the whole antibody, 27C1/10A6 IgG4 (specific for TGF β_1), 27C1 VH DNA was prepared from the clone isolated above, in example 1. The VH gene was amplified by PCR using the oligonucleotides VH3BackSfiEu and VHJH6ForBam (Table 1) with cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C. Following digestion with SfiI and BamHI, the VH gene was cloned into the vector vh cassette2 (Figure 5) digested with SfiI and BamHI. Ligated DNA was transformed into E. coli TG1. Ampicillin resistant colonies were obtained and those containing the correct insert identified by DNA sequencing.

20 Plasmid DNA from these colonies was prepared and the DNA digested with HindIII and BamHI. The HindIII-BamHI restriction fragment was ligated into the human IgG4 heavy chain expression vector pG4D100 (Figure 6), which had been digested with HindIII and BamHI and the DNA transfected into E.coli TG1 by electroporation. The sequence of the VH gene insert was again verified by DNA sequencing.

25 For the light chain, the VL gene of 10A6, isolated in example 1, was first mutagenized to remove its internal BamHI site using site directed mutagenesis (Amersham RPN1523) with the oligonucleotide DeltaBamHI (Table 1). The resulting

VLD_{Bam}H1 gene was amplified by PCR using the oligonucleotides Vλ3/4BackEuApa and HuJλ2-3ForEuBam (Table 1). Following digestion of the amplified insert with ApaI and BamHI, the VL gene was cloned into the vector vlcassetteCAT1 (Figure 7) digested with ApaI and BamHI. Ligated DNA was 5 transformed into E.coli TG1. Ampicillin resistant colonies were obtained and those containing the correct insert were identified by DNA sequencing.

Plasmid DNA from these colonies was prepared and the DNA digested with Hind III and BamHI. The HindIII-BamHI restriction fragment containing the leader sequence and the VL domain was ligated into the human lambda light chain expression vector, 10 pLN10 (Figure 8), which had been digested with HindIII and BamHI. Following electroporation, transformants in E.coli were checked by DNA sequencing.

Plasmid DNA was prepared from the pG4D100-27C1 clone and the pLN10-10A6 clone. This DNA was then co-transfected into DUKXB11 Chinese Hamster Ovary 15 (CHO) cells by electroporation (290V; 960μF). The cells were then grown for 2 days in non-selective medium (alpha-MEM plus nucleosides). Cells were then transferred to a selective medium (alpha-MEM plus 1mg/ml G418 without nucleosides) and grown in 96 well plates. Colonies were then transferred to 24 well plates and samples assayed by sandwich ELISA for assembled human IgG4 antibody and by binding to 20 TGFbeta1 in ELISA (as in example 1). For the sandwich ELISA, goat anti-human IgG coated on to the ELISA plate and captured human IgG4 detected using goat anti-human lambda light chain alkaline phosphatase conjugate. High expressing cell lines were then derived by amplification of the inserted genes using selection in the presence of methotrexate (R.J. Kaufman Methods Enzymol. 185 537-566, 1990).

The whole antibody 6H1 IgG4 (specific for TGFbeta2) was constructed in a similar way to the above construction of 27C1/10A6 IgG4. The 6H1 VH gene (example 2) 25 was cloned into pG4D100 as for 27C1 above except that PCR amplification was performed with the oligonucleotides VH3BackSfiEu and VHJH1-2FORBam. The 6H1 VL gene (example 2) was subcloned into vlcassetteCAT1 as above except that PCR amplification was performed with the oligonucleotides Vk2BackEuApa and 30 HuJk3FOREuBam. However, since the 6H1 VL is a kappa light chain the

HindIII-BamHI fragment was subcloned into the human kappa light chain expression vector pKN100 (Figure 9) which had been digested with HindIII and BamHI. High expressing cell lines were then isolated as described above. Clones expressing antibody were identified from culture plates by sandwich ELISA for assembled human IgG4 antibody (detected using goat anti-human kappa light chain conjugate and by binding to TGFbeta2 in ELISA (as in example 2).

To construct the whole antibodies 6A5 IgG4 and 6B1 IgG4, the same 6H1 VH construct in pG4D100 was used as for 6H1IgG4 since these antibodies all have the same VH gene. The 6B1 and 6A5 genes were each subcloned into vlcassetteCAT1 as above for the 10A6 light chain except that PCR amplification was performed with the nucleotides Vλ3backEuApa and HuJλ2-3ForEuBam. The HindIII-BamHI restriction fragment was then subcloned into pLN10 as above. Clones expressing antibody were identified from culture plates by sandwich ELISA for assembled human IgG4 antibody (detected using goat anti-human kappa light chain conjugate and by binding to TGFbeta2 in ELISA (as in example 2).

Properties of whole antibody constructs

20 Purification of whole antibodies

Serum-free supernatant from CHO cells producing the relevant IgG was clarified by centrifugation at 8000 rpm (Beckman JS2-21) prior to purification. The supernatant was applied to a HiTrap Protein A Sepharose prepacked affinity column from Pharmacia, either 1 or 5ml size, with binding capacities of 25 or 120 mg respectively. Each IgG had a dedicated column to avoid any potential carry over of material from one purification to another. The column was equilibrated to phosphate buffered saline (PBS) with ten column volumes of 1xPBS prior to applying the supernatant. When all the supernatant had been applied to the column at a flow rate of 2-4 ml/minute, again, depending on the column size, the column was washed with ten column volumes of 1xPBS to remove any non-specifically bound material. Elution of the bound protein was achieved using 0.1M sodium acetate, adjusted to pH 3.3 with glacial acetic acid.

The eluted material was collected in 8 fractions of 1.5 ml volume, and the amount of protein determined by measuring the absorbance at 280nm, and multiplying this value by 0.7 to get a value in mg/ml. This was then neutralised with 0.5ml of 1M Tris.HCl pH 9.0 per 1.5ml fraction, and the protein-containing fractions pooled and dialysed against 1x PBS to buffer exchange the IgG. The column was returned to neutral pH by running ten column volumes of 1xPBS through, and was stored in 20% ethanol as a preservative until required again.

5 A sample was then run on 10-15% SDS-PAGE (Phast system, Pharmacia) and silver stained. this allowed an assessment of the purity of the IgG preparation. This was

10 usually found to be about 80-90%, with only a couple of other bands prominent on the stained gel.

Binding specificity by ELISA

15 The IgG4 antibodies 6B1 and 6A5 were shown to bind TGFbeta2 with very low cross-reactivity to TGFbeta1 and TGFbeta3 and no detectable cross-reactivity with a range of non-specific antigens: interleukin-1; human lymphotoxin (TNF β); human insulin; human serum albumin; single stranded DNA; oxazolone-bovine serum albumin; keyhole limpet haemocyanin; chicken egg white trypsin inhibitor; chymotrypsinogen; cytochrome c; glyceraldehyde phosphate dehydrogenase; ovalbumin; hen egg lysozyme; bovine serum albumin and tumour necrosis factor α - (TNF α) (Figure 13(a) and (b)). Likewise the antibodies 6B1, 6A5 and 6H1 IgG4 bound strongly to TGFbeta2 coated on a BIACore sensor chip but not significantly to TGFbeta1 or TGFbeta3 coated chips.

Binding properties of whole antibodies by BIACore

30 The affinity constants of the above antibodies were determined by BIACore, using the method of Karlsson et al. J. Immunol. Methods 145, 299-240, 1991 (supra) and found to be approximately 5nM for 27C1/10A6 IgG4 for TGFbeta1 and 2nM for 6H1 IgG4 for TGFbeta2. The antibody 27C1/10A6 IgG4 also shows some cross-reactivity with

TGFbeta2 coated onto Biosensor chips but the dissociation constant is approximately 10 fold or more higher for TGFbeta2 compared to TGFbeta1. There was no significant cross-reactivity with lysozyme coated onto a BIACore sensor chip.

- 5 Neutralisation and inhibition of radioreceptor binding by IgG4 antibodies to TGFbeta1 and TGFbeta 2 is described in examples 3 and 4.

Example 3 Neutralisation by Antibodies of the Inhibitory Effect of TGF b1 and TGF b2 on Cell Proliferation

The neutralising activity of the antibodies described in examples 1 and 2 were tested
5 in a modification of a bioassay for TGF β as described by Randall et al (1993) J.
Immunol Methods 164, 61-67. This assay is based on the ability of TGF β_1 and TGF β_2 to inhibit the interleukin-5 induced proliferation of the erythroleukaemia cell line,
TF1 and being able to reverse this inhibition with specific TGF β antibodies.

10 Method

Cells and maintenance

15 The human erythroleukaemia cell line TF1 was grown in RPMI 1640 medium
supplemented with 5% foetal calf serum, penicillin/streptomycin and 2ng/ml
rhGM-CSF in a humidified incubator containing 5% CO₂ at 37°C. Cultures were
passaged when they reached a density of 2×10^5 /ml and diluted to a density of 5×10^3 /ml.

20 Cytokines and Antibodies

rhGM-CSF and rhIL-5 were obtained from R&D systems, rhTGF β_2 was obtained
AMS Biotechnology. Rabbit anti TGF β_2 antibody was from R&D Systems and
Mouse anti-TGF $\beta_{1,2,3}$ was from Genzyme. Other antibodies against TGF β_2 were as
25 described in examples 1&2.

Titration of Inhibition of Proliferation by TGF β_2

30 Doubling dilutions of TGF β_2 (800pM - 25pM) for the construction of a dose response
curve were prepared on a sterile microtitre plate in 100 μ l of RPMI 1640 medium
containing 5% foetal calf serum and antibiotics. All dilutions were performed at least

in quadruplicate. Additional wells containing 100 μ l of the above medium for reagent and cells only controls were also included.

5 TF1 cells were washed twice in serum free RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 5% foetal calf serum, 100U/ml penicillin and 100 μ g/ml streptomycin and 4ng/ml rhIL-5 at a density of 2.5×10^5 /ml. Aliquots of 100 μ l were added to the previously prepared dilution series and the plate incubated for 48hr. in a humidified incubator containing 5% CO₂ at 37°C.
10 Cell proliferation was measured colourimetrically by addition of 40 μ l CellTiter 96 substrate (Promega), returning the plate to the incubator for a further 4hr and finally determining the absorbance at 490nm. The percentage inhibition for each concentration of TGF β_2 as compared to cell only wells was then calculated.

Assay for Neutralisation of TGF β_2 Inhibitory Activity by Anti-TGF β_2 Antibodies

15 Neutralisation of TGF β_2 was determined by making doubling dilutions in of each purified antibody in 100 μ l of medium as above. TGF β_2 was added to each antibody dilution to give a final concentration equivalent to that which gave 50% inhibition in the titration described above. Each dilution was prepared in quadruplicate. Additional 20 wells were prepared for antibody only, cells only and reagent controls. Cell preparation and determination of cell proliferation was performed as described above.

Results

25 TGF β_2 was shown to inhibit the proliferation of TF1 cells by 50% at a concentration of 50pM. This concentration was used for all neutralisation experiments.

These assays showed that TGF β_2 activity was neutralised in a dose dependant 30 manner for both scFv fragments (figure 10) and for whole IgG4 antibodies (figure 11). The concentration of antibody which gave 50% inhibition was determined from the graphs and is shown in table 4.

Example 4 Inhibition by antibodies of TGF β binding to receptors measured in a radioreceptor assay

5 Single chain Fv fragments and whole IgG4 antibodies from different clones were expressed and purified and their ability to inhibit binding of TGF β to receptors measured in a radioreceptor assay.

PURIFICATION OF scFv

10

ScFvs containing a poly histidine tail are purified by immobilised metal affinity chromatography. The bacterial clone containing the appropriate plasmid is inoculated into 50 ml 2TY medium containing 2% glucose and 100 μ g/ml ampicillin (2TYAG) and grown overnight at 30°C. The next day the culture is added to 500 ml prewarmed

15

2TYAG and grown at 30°C for 1 h. The cells are collected by centrifugation and added to 500 ml 2TY containing ampicillin and 1 mM IPTG and grown at 30°C for 4 h. The cells are then collected by centrifugation and are resuspended in 30 ml ice-cold 50 mM Tris HCl pH 8.0, 20% (w/v) sucrose, 1 mM EDTA. After 15 min end-to-end mixing at 4°C the mixture is centrifuged at 12 k rpm for 15 min at 4°C.

20

The supernatant is removed and to it added ~ 1ml NTA-agarose (Qiagen 30210) and mixed at 4°C for 30 min. The agarose beads are washed extensively with 50 mM sodium phosphate, 300 mM NaCl and loaded into a small column. After further washing with 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole pH 7.4

25

scFv is eluted with 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 7.4. 0.5 ml fractions are collected and the protein containing fractions identified by measuring the A_{280nm} . Pooled fractions are concentrated and scFv further purified by gel filtration in PBS on a Superdex 75 column (Pharmacia).

30

PURIFICATION OF WHOLE ANTIBODIES

Whole IgG4 antibodies were purified as described in example 2.

RADIORECEPTOR ASSAY FOR TGF- β

Neutralisation of TGF- β activity is measured by the ability of the scFvs and IgGs to inhibit the binding of 125 I labelled TGF- β to its receptors on A549 human lung carcinoma cells.

A549 cells (ATCC CCL 185) are grown in high glucose Dulbecco's modified Eagle's medium (Sigma D-6546) supplemented with 10% foetal calf serum (PAA), 2 mM glutamine (Sigma G-7513), penicillin/streptomycin (Sigma P-0781), MEM non-essential amino acids (Sigma M-7145).

Cells are seeded at 1-2 x 10⁵ cells / ml / well into the wells of 24-well cluster plates and grown for 24 h in serum-free DMEM. Cell monolayers are washed twice with serum-free DMEM and 0.5 ml binding medium (DMEM/Hams F12 (Sigma D-6421) containing 0.1% (v/v) BSA added to each well.

Aliquots of 125 I-TGF- β 1 or - β 2 (70-90 TBq/mmol; Amersham International) at 20 pM are preincubated with antibody in binding medium at room temperature for 1 h. Duplicate samples of 0.5 ml of TGF- β /antibody mixtures are then added to the cell monolayers and are incubated at 37°C for 1-2 h. Control wells contain TGF- β only. Unbound TGF- β is removed by washing 4 times with Hank's balanced salt solution containing 0.1% BSA. Cells are solubilised in 0.8 ml 25 mM Tris HCl pH 7.5, 10% glycerol, 1% Triton X-100 at room temperature for 20 min. The contents of each well are removed and 125 I measured in a gamma counter. The potency of each scFv or IgG is measured by the concentration of antibody combining sites necessary to inhibit binding of TGF- β by 50% (IC₅₀; Table 5). Thus the IC₅₀ values are below 10nM and in some cases below 1nM indicating very potent antibodies.

Example 5 Prevention of Scar Formation by Antibodies Against TGF b1 and TGF b2 in the Injured Central Nervous System of the Rat

5 Logan *et al* (1994) Eur.J Neuroscience 6,355-363 showed in a rat model of CNS injury, the ameliorating effect of a neutralising turkey antiserum directed against TGF β_1 on the deposition of fibrous scar tissue and the formation of a limiting glial membrane that borders the lesion. A study was set up to investigate the effects of neutralising engineered human antibodies directed against both TGF β_1 and TGF β_2 in the same rat model. The derivation of the antibodies used in this study is described
10 in examples 1 and 2.

Method

Animals and surgery

15 Groups of five female Sprague-Dawley rats (250g) were anaesthetised with an i.p. injection. The anaesthetised rats had a stereotactically defined lesion made into the right occipital cortex (Logan *et al* 1992 Brain Res. 587, P216-227) and the lateral ventricle was surgically cannulated and exteriorised at the same time (Logan *et al* 20 1994 supra).

Neutralisation of TGF β

25 Animals were intraventricularly injected daily with 5 μ l of purified anti TGF β antibodies (Table 3) diluted in a vehicle of artificial cerebrospinal fluid as described by Logan *et al* 1994 supra. Fourteen days post lesion all animals were perfusion fixed and 7mm polyester wax sections were processed for histochemical evaluation of the lesion site by immunofluorescent staining.

30

Fluorescent immunohistochemistry and image analysis

Morphological changes within the wound site were followed by immunofluorescent staining with antibodies to fibronectin and laminin detected with anti-species FITC conjugates (Logan *et al* 1994 *supra*). These changes were semi-quantitatively assessed by image analysis using a Leitz confocal microscope linked to a Biorad MRC500 laser scanning system. Readings were taken at standard positions mid-way along the lesion.

10

ResultsEffects of antibodies to TGF β at the site of CNS injury

15 Quantitation of the specific relative fluorescence for each of the antibodies is shown in figure 12 a and b. Laminin is a measure of the formation of the glial limitans externa along the boundaries of the wound and together with fibronectin forms a matrix of fibrous tissue within the centre of the wound. Quantitation by image analysis of these two proteins allows the degree of scarring at the wound site to be
20 determined.

Compared with the saline control (fig.12 a,b), There is a considerable decrease in fibronectin and laminin immuno-localisation in the wound in the anti-TGF β antibody treated brains. Thus this indicates that these engineered human antibodies directed against epitopes on TGF β_1 & TGF β_2 ameliorate the effects of injury to the CNS both separately and together by preventing the deposition of the cellular matrix proteins fibronectin and laminin within the wound site. Previously Logan *et al* (1994 *supra*) had shown the effectiveness of a polyclonal turkey anti-sera directed against TGF β_1 . This is the first report of any antibodies directed against TGF β_2 having been shown
25 to be effective in this model.
30

Example 6 Suppression of experimental glomerulonephritis using human antibodies against human TGF β

The ability of human antibodies against human TGF β to neutralise TGF β activity, 5 and thus prove beneficial in the treatment of fibrotic disease, was tested in an animal model of the kidney disease, glomerulonephritis.

Antibodies directed against TGF β_1 have been shown to be effective in the suppression of experimental glomerulonephritis (W.A. Border et al Nature 346 10 371-374, 1990) and other fibrotic diseases (W.A. Border & N.A. Noble New Engl. J. Med. 331 1286-1292, 1994). In this example, it is shown that antibodies directed against either TGF β_1 or TGF β_2 are effective in the treatment of glomerulonephritis. Induction of glomerulonephritis in rats with a single injection of anti-thymocyte serum was followed by treatment with an injection of either 15 antibody directed against TGF β_1 or of saline.

31G9 and 6A5 scFv (example 1) were expressed using a T7 polymerase controlled vector system (J.H. Christensen et al FEBS Lett. 281 181-184, 1991). Active scFv protein was prepared from inclusion bodies using the methodology described in 20 WO94/18227 (H.C. Thøgersen et al). The scFv preparations were homogeneous as determined by SDS-PAGE and by gel filtration chromatography on Superose 12.

Five groups of rats were used:-

Group A: Normal controls, no anti-thymocyte serum treatment
25 Group B: Disease control (saline treatment)
Group C: Treatment daily with 25 μ g 31G9 single chain Fv (anti-TGF β_1)
Group D: Treatment daily with 25 μ g 6A5 single chain Fv (anti-TGF β_2)
Group E: Treatment daily with 25 μ g 31G9 and 8 μ g 6A5 single chain Fv
30 Groups B to E each received a dose of 0.25ml sheep anti-thymocyte serum (ATS; Border et al, 1990 supra). One hour after ATS injection, each group received 200 μ l PBS (group B) or the appropriate antibody (200 μ l in PBS). On days 1 to 5,

these doses were repeated for groups B to E. On day 6, all rats were sacrificed.

Urinary protein was measured (a measure of glomerular injury: J.M. Ginsberg et al New Engl. J. Med. 309 1543-1550, 1983) for 24h on days 5 to 6 and was found to be significantly lower for the rats treated with 6A5 scFv than for the disease control (see Figure 14). The extent of glomerular injury was determined by examination of glomeruli stained with periodic acid-Schiff's base (30 glomeruli for each rat). These glomeruli are scored for the extent of glomerular matrix accumulation (30 glomeruli for each rat) on histological examination of stained sections (Border et al, 1990 supra; W.A. Border et al Nature 360 361-364, 1992). Scoring was performed by two independent scientists for each rat. There was a significantly lower increase in extracellular matrix deposition for the 6A5 scFv treated rat compared to the disease control (Figure 15). There was also a somewhat lower increase for 31G9 scFv but this difference in deposition was not statistically significant.

Hence the human antibody against human TGF β_2 is effective in suppression of experimental glomerulonephritis.

Table 1: Oligonucleotide primers used in the identification and characterisation of TGF- β 1 antibodies.

Primer	Nucleotide sequence 5' to 3'
182 mutVHCDR3	5' CGT GGT CCC TTT GCC CCA GAC GTC CAC ACC ACT AGA ATC GTC ACT ATA TTC CCC AGT TCG CGC ACA GTA ATA CAC AGC CGT
pUC19reverse	5' AGC GGA TAA CAA TTT CAC ACA GG 3'
fdtet seq	5' GTC GTC TTT CCA GAC GTC AGT 3'
PCR-H-Link	5' ACC GCC AGA GCC ACC TCC GCC 3'
PCR-L-Link	5' GGC GGA GGT GCC TCT GCC GGT 3'
myc seq 10	5' CTC TTC TGA GAT GAG TTT TGG 3'
HuJH4-5For	5' TGA CGA GAC GGT GAC CAG GGT TCC 3'
RL1	5' G(C/A)A CCC TGG TCA CCG CCT CA GGT GGA GGC GGT TCA GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'
RL2	5' GGA CAA TGG TCA CCG TCT CTT CA GGT GGA GGC GGT TCA GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'
RL3	5' GGA CCA CGG TCA CCG TCT CCT CA GGT GGA GGC GGT TCA GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'

VH1b/7a back **Sfi**
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (AG)TG CAG CTG GTG CA(AG) TCT GG-3'

VH1c back **Sfi**
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC (GC)AG GTC CAG CTG GT(AG) CAG TCT GG-3'

54

VH2b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG (AG)TC ACC TTG AAG GAG GAC (AG)TC TCT GG-3'

VH3b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG (GC)AG GTG CAG CTG GTG GAG TCT GS-3'

VH3c back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTG CAG CTG GAG (AT)C (TC) GG-3'

VH4b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTG CAG CTG CAG TGG GG-3'

VH4c back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG (GC)TG CAG CTG CAG GAG TC(GC) GG-3'

VH5b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GAA(AG) GTG CAG CTG GTG CAG CAG TCT GG-3'

VH 6a back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GAA CAG CTG CAG CAG TCA GG-3'

VH3BACKSFIEU 5'- AGC TCG GTC CTC GCA ACT GCG GCC CCT GGG GCC CAC AGC GAG GTG CAG CTG GTG GAG TCT GG - 3'

VHJH6FORBam 5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CGT GGT CCC - 3'

DeltaBamHI 5'-GA GAA TCG GTC TGG GAT TCC TGA CGG CCG G-3'

VH3/4BackEuapa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC CAC GTT ATA CTG ACT CAG GAC CC -3'

HuJλ2-3ForEuBam 5'-G GTC CTC GCA ACT GCG GAT CCA CTC ACC TAG GAC GGT CCC- 3'

VHJH1-2FORBam 5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CAG GGT GCC - 3'

V\2BackEuApa 5' - AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC GAT GTT GTG ATG ACT CAG TCT CC-3'
HuJXForEuBam 5' - G GTC CTC GCA ACT GCG GAT CCA CTC ACG TTT GAT ATC CAC TTT GGT CCC -3'
V\3BackEuApa 5' - AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC TCG TCT GAG CTG ACT CAG GAC CC -3'

Table 2 Properties of single chain Fv fragments for binding to TGFbeta1 or TGFbeta2 determined using BIACore

<i>Antibody</i>	<i>k_{off} (s⁻¹)</i>	<i>K_d(nM)</i>
<u>TGFbeta1</u>		
31G9	9.0 × 10 ⁻⁴	12
CS32	1.2 × 10 ⁻³	
CS39	1.7 × 10 ⁻³	
<u>TGFbeta2</u>		
6A5	1.4 × 10 ⁻⁴	0.7
6B1	6.0 × 10 ⁻⁴	
6H1	1.1 × 10 ⁻³	
14F12	2.1 × 10 ⁻³	

Table 3 Daily dose levels for individual animals in each group

Group	Clone	Antibody format	Antigen	Dose
1	Saline Control	-	-	-
2	31G9	scFv	TGF β_1	20ng
3	6A5	scFv	TGF β_2	20ng
4	27C1/10A6	IgG4	TGF β_1	692ng
5	6H1	IgG4	TGF β_2	1.76 μ g
6	31G9 +6A5	scFv's	TGF β_1 TGF β_2	20ng "
7	27C1/10A6 + 6H1	IgG4's	TGF β_1 TGF β_2	692ng 1.76 μ g

Table 4 I.C.₅₀ values for antibodies in TF1 assay

Antibody	scFv (nM)	IgG4 (nM)
6H1	1.5	100
6B1	15	11
6A5	8	150
14F12	90	nd

nd = not determined

Table 5 IC₅₀ values for antibodies measured using a radioreceptor assay.

Anti-TGF-β1 antibody	IC ₅₀ , nM
7A3 scFv	>100
31G9 scFv	30
CS32 scFv	4.5
CS39 scFv	~60
27C1/10A6 IgG	9
VT37 scFv	~100

Anti-TGF-β2 antibody	IC ₅₀ , nM
6A5 scFv	1.5
6A5 IgG	~6
6B1 scFv	0.3
6B1 IgG	0.6
6H1 scFv	0.22
6H1 IgG	~10
11E6 IgG	1.6
14F12 scFv	3
VT37 scFv	2

Figure 1 Sequences of antibodies specific for TGF β 1

[a] Antibodies to TGF β 1 elicited directly from repertoire

(i) LB2 VH [also known as TAS VH]

Sequence Range: 1 to 369

10	20	30	40				
*	*	*	*	*	*	*	*
CAG CTC CAN CTG CTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG							
Q V O L V E S G G V Q P G R >							
a a a a TRANSLATION OF 7A3 VH.SEQ [A] a a a a >							
50	60	70	80	90			
*	*	*	*	*	*	*	*
TCC CTC AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT							
S L R L S C A A S G F T F S S Y >							
a a a a TRANSLATION OF 7A3 VH.SEQ [A] a a a a >							
100	110	120	130	140			
*	*	*	*	*	*	*	*
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG EGG CTG GAG TGC GTG							
G M H W V R Q A P G K L E W V >							
a a a a TRANSLATION OF 7A3 VH.SEQ [A] a a a a >							
150	160	170	180	190			
*	*	*	*	*	*	*	*
GCA GTT ATA TCA TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTC							
A V I S Y D G S N K Y Y A D S V >							
a a a a TRANSLATION OF 7A3 VH.SEQ [A] a a a a >							
200	210	220	230	240			
*	*	*	*	*	*	*	*
AAAG GCG CGA TTC ACC ATC TCC AGA GAC ATT TCC AAG AAC ACG CTG TAT							
K G R F T I S R D N S K N T L Y >							
a a a a TRANSLATION OF 7A3 VH.SEQ [A] a a a a >							

1/43

2143

1A-E5 VI

Sequence Range: 1 to 345

3/43

LA-H6 VH

Sequence Range: 1 to 354

5143

a_a_a_a_TRANSLATION OF 1AH-6 VH [A]		a_a_a_a_TRANSLATION OF 1AH-6 VH [A]	
290	300	310	320
*	*	*	*
ACG TCT GAT CCT TTA CGC TAT CCT ATT GAC TAC TGG GGC CAG GGA ACC			
T S D P L R Y P I D Y W G Q G T>			
a_a_a_a_TRANSLATION OF 1AH-6 VH [A]	a_a_a_a_TRANSLATION OF 1AH-6 VH [A]		
330	340	350	+
370	380	390	
GTC GTC ACC GTC TCG AGT			
L V T V S S>			
TRANSLATION OF 1_____			

(iv) 31C9 VH

Sequence Range: 1 to 369

6143

GAC	ATC	GTG	ATG	ACC	CAG	TCT	CCT	RCC	ACC	CTG	TCT	GCA	TCT	GTA	GGA
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
10			20					30				40			

(v) 3169 WL

a a a a TRANSLATION OF 31G9 VL. SEQ [A] -

Figure 1 (b) Light chains of antibodies to TGFbeta1 isolated by chain shuffling

(i) 7A3 VL

Sequence Range: 1 to 342

89/43
10 * 20 * 30 * 40 *
GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT GTG TCT CTG GGC
D I V N T Q S P D S L A V S L G>
a a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a a >

50 * 60 * 70 * 80 * 90 *
GAG AGG GCC ACC ATC AAC TGC AAG TCC AGC CAG AGT CTT TTA TAC AGC
E R A T I N C K S S Q S L Y S>
a a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a a >

100 * 110 * 120 * 130 * 140 *
TAC AAC AAG ATG AAC TAC TTA GCT TGG TAC CAG CAG AAA CCA GGA CAG
Y N K M N Y A W Y Q O K P G Q>
a a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a a >

150 * 160 * 170 * 180 * 190 *
CCT CCT AAG CTC ATT AAC TGG GCA TCT ACC CGG GAA TCC GGG GTC
P P K L L T N W A S T R E S G V>
a a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a a >

200 * 210 * 220 * 230 * 240 *
CCT GAC CGA TTC ACT GGC AGC GGG TCT GGG ACA GAT TTC ACT CTC ACC
P D R F S G S G T D F T L T>

9/43

a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a >

250	*	260	*	270	*	280	*
I	S	S	L	Q	A	E	D
ATC	AGC	AGC	CTG	CAG	GCT	GAA	GAT
GCA	GAT	GTC	GCA	GTT	GCA	TAT	TAC
CAA	Q>					TGT	TGT
<u>a a a</u>							

a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a >

290	*	300	*	310	*	320	*	330	*	340	*
Y	Y	A	T	P	L	T	F	G	H	G	T
TAT	TAT	GCA	ACT	CCT	CTG	ACG	TTC	GGC	CAC	GGG	AAG
<u>a a a</u>											

a a a TRANSLATION OF 7A3 VL. SEQ [A] a a a >

AAA CGT
K R>
a a a >

(ii) 10A6 VL

Sequence Range: 1 to 357

a a a TRANSLATION OF 10A6 VL. SEQ [A] a a a >

10	*	20	*	30	*	40	*
H	V	I	L	T	Q	D	P
CAC	GTT	ATA	CTG	ACT	CAG	GAC	CCT
<u>a a a</u>							

a a a TRANSLATION OF 10A6 VL. SEQ [A] a a a >

50	*	60	*	70	*	80	*	90	*
T	V	R	I	T	C	Q	G	D	S
ACA	GTC	AGG	ATC	ACG	TGC	CAA	GGA	GAC	AGC
<u>a a a</u>									

a a a TRANSLATION OF 10A6 VL. SEQ [A] a a a >

10/43

AGT TGG TAC CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT							
S W Y Q Q K P G Q A P V L V I Y>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 10A6 VL. SEQ [A]_a_a_a_a_a_a>							
150 * * * * *	160 *	170 *	180 *	190 *			
GGT GAA AAC AGC CGG CCC TCC GGG ATC CCA GAC CGA TTC TCT GGC TCC							
G E N S R P S G I P D R F S G S>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 10A6 VL. SEQ [A]_a_a_a_a_a_a>							
200 * * * * *	210 *	220 *	230 *	240 *			
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GCG GCT CAG GCG GAA							
S S G N T A S L T G A Q A E>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 10A6 VL. SEQ [A]_a_a_a_a_a_a>							
250 * * * * *	260 *	270 *	280 *				
GAT GAA GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT ACC CAT							
D E A D Y Y C N S R D S G T H>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 10A6 VL. SEQ [A]_a_a_a_a_a_a>							
290 * * * * *	300 *	310 *	320 *	330 *			
CTA GAA GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA CGT							
L E V F G G T K L T V L G							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 10A6 VL. SEQ [A]_a_a_a_a_a_a>							

Figure 1 (c) Antibodies to TGF β 1 isolated from CDR3 spiking experiment

(i) 27C1 VH

Sequence Range: 1 to 369

10 20 30 40

11/43

* * * * * CAG GTG CAA CTG GTG GAG TCT GGG GGA GGC GTC CCT CAG CCT GGG A3G
Q V Q L V E S G G V V Q P G R>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

50 * * 60 * * 70 * * 80 * * 90 *
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA CTC ACC TTC AST AGC TAT
S L R L S C A A S G L T F S S Y>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

100 110 120 130 140 *
GAC ATG CAC TGG GTC CGC CAG CCT CCA GCC AAG GGG CTG GAG TGG GTG
D M H W V R Q P A K G L E W V>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

150 * * 160 * * 170 * * 180 * * 190 *
GCC GTT ATA TCA TAT GAT GGA AGT AGT AAA TAC TAT GCA GAC TCC GTG
A V I S Y D G S S R Y Y A D S V>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

200 210 220 230 240 *
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K N T L Y>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

250 * * 260 * * 270 * * 280 *
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
L Q M N S L R A E D T A V Y Y C>
a a a a TRANSLATION OF 27C1 VH.SEQ [A] a a a a >

290 * * 300 * * 310 * * 320 * * 330 *
* * * * *

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340 * 350 * * 360
 TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA
 W G Q G T T V T V S S >
 D TRANSLATION OF 27C1 VII SEQ [A] _____ a _____>

13/43

Figure 2 Sequences of antibodies specific for EGGbeta2

¹Antibodies to IgGbeta 2 isolated directly from repertoires

2A-H11 VH (also known as 6H1 VH)

Sequence Range: 1 to 345

14/43

(ii) Zn-Ag also known as 11E6 VTH

5/43

GCT ATG CAC TGG GTC CGC CAG CCT CCA GCC AAG GGG CTG GAG TGG GTG							
A M H W V R Q A P A K G L E W N V>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 11E6 VH.SEQ [A]_a_a_a_a_a_a>							
150 * * * 160 * * * 170 * * * 180 * * * 190 * * *							
GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG							
A V I S Y D G S N K Y A D S V>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 11E6 VH.SEQ [A]_a_a_a_a_a_a>							
200 * * * 210 * * * 220 * * * 230 * * * 240 * * *							
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT							
K G R F T I S R D N S K N T L Y>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 11E6 VH.SEQ [A]_a_a_a_a_a_a>							
250 * * * 260 * * * 270 * * * 280 * * *							
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCC GTG TAT TAC TGT							
L Q M N S L R A E D T A V Y Y C>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 11E6 VH.SEQ [A]_a_a_a_a_a_a>							
290 * * * 300 * * * 310 * * * 320 * * * 330 * * *							
GCA AGA GCG GCG TTG GAA ACG ACG TGG GGC CAA GGA ACC CTG GTC ACC							
A R A G L E T T W G Q G T L V T>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION OF 11E6 VH.SEQ [A]_a_a_a_a_a_a>							
340 * * * 350 * * *							
GTC TCC TCA AGT GG							
V S S S G>							
<u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> TRANSLATION_a_a_a_a_a_a>							

(iii) Gold11-VH

Sequence Range: 1 to 359

16143

13 | 43

Goldilocks

18/43

a a a TRANSLATION OF GCLD11-VL [A] a a a >

150	160	*	170	*	*	180	*	190							
*	*	*	*	*	*	*	*	*							
TAT	GGC	AAU	GAT	CAG	CGG	CCC	TCA	GGG	ATC	CCT	GAC	CGA	TTC	TCT	
I	Y	G	N	D	Q	R	P	S	G	I	P	D	R	F	S>
a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a >
200	210	*	220	*	*	230	*	240							
*	*	*	*	*	*	*	*	*							
TTC	TTT	AAU	TCT	CCG	ACC	TCA	GCC	TCC	CTG	GCC	ATC	ACT	GGG	GTC	CAG
G	S	K	S	G	T	S	A	S	L	A	T	G	V	Q>	
a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a >
250	260	*	270	*	*	280	*	*							
*	*	*	*	*	*	*	*	*							
GCT	GAA	GAC	GAG	GCT	GAC	TAT	TAC	TGC	CAG	TCA	TAT	GAC	AGC	AGC	CTG
A	E	D	E	A	D	Y	Y	C	Q	S	Y	D	S	S	L>
a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a >
290	300	*	310	*	*	320	*	330							
*	*	*	*	*	*	*	*	*							
ACG	GGT	TCC	AGG	GTC	TTC	GGA	ACT	GGG	ACC	AAG	GTC	ACC	GTC	CTA	GST
R	G	S	R	V	F	G	T	G	T	K	V	T	V	L	G>
a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a >

(V) -G2

Sequence Range: 1 to 381

10	20	*	30	40											
*	*	*	*	*											
CAG	GTA	CAA	CCT	CAG	CAG	TCT	GSG	GGA	GAG	GTG	AAG	CAG	CCT	GGG	GGC
Q	V	Q	P	Q	Q	S	G	G	E	V	K	Q	P	G	A>
a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a	a a a >
50	60	70	80	90											

(v)
1-H6

Sequence Range: 1 to 381

CAT ATC TGG GGC CAA GGG ACA ATG GTC ACC GTC ACC GTC TCT TCA
D I W G Q G T M V T V S P S>
a a a a TRANSLATION OF 1-G2-VH [A] a a a a >

20/43

10	*	*	20	*	*	30	*	*	40	*	*				
GAG	GTG	CAG	CTG	GTG	GAG	TCT	GGG	GGA	GGC	GTG	CAG	CCT	GGG	AGG	
E	V	Q	L	V	E	S	G	G	V	V	Q	P	G	R>	
a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	
50	*	*	60	*	*	70	*	*	80	*	*	90	*	*	
TCC	CTG	AGA	CTC	TCC	TGT	GCA	GCG	TCT	GGA	TTC	ACC	TTC	AGG	AAC	TAT
S	L	R	L	S	C	A	A	S	G	F	T	F	R	N	Y>
a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	>
100	*	*	110	*	*	120	*	*	130	*	*	140	*	*	
GSC	ATG	CAC	TGG	GTC	CGC	CAG	GCT	CCA	GGC	AAG	GGG	CTG	GAG	TGG	GTC
G	M	H	W	V	R	Q	A	P	G	K	G	L	E	W	V>
a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	>
150	*	*	160	*	*	170	*	*	180	*	*	190	*	*	
GCA	GTT	ATA	TGG	TAT	GAT	GGA	AGT	AAT	AAA	TAC	TAT	GCA	GAC	TCC	GTC
A	V	I	W	Y	D	G	S	N	K	Y	A	D	S	V	>
a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	>
200	*	*	210	*	*	220	*	*	230	*	*	240	*	*	
AAG	GGC	CGA	TTC	ACC	ATC	TCC	AGA	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT

Figure 2(b) Light chains of antibodies specific for TGF β 2 isolated following chain shuffling

	10	*	*	20	*	*	30	*	*	40	*	*			
GAA	GTT	GTG	CTG	ACT	CAG	TCT	CCA	TCC	TCC	TCT	GCA	TCT	GTA	GGA	
E	V.	V	L	-	Q	S	P	S	S	L	S	A	S	V	G>
a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	
50	60	70	70	80	80	90	90								

Sequence Range: 1 to 324

5

22/43

665 V.L.

Sequence Range: 1 to 32?

23/43

24/43

D	E	A	D	Y	C	S	R	D	S	S	G	N	H>
<u>a</u>													

TRANSLATION OF 6A5 VL. SEQ [A] aaaa>

290 * 300 * * * * * * * * * * * *

V V F G G T K L T V L G>

a

TRANSLATION OF 6A5 VL. SEQ [A] aaaa>

(W) 6B1..VI.

Sequence Range: 1 to 330

10	20	30	40
*	*	*	*
T(C); TCT GAC CTC ACT CAG GAC CCT GCT GTC TCT GTG GCC TTG CGA CAG	A	V	S
G	E	L	T
<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>

TRANSLATION OF 6B1 VL. SEQ [A] aaaa>

* * * * * * * * * * * * *

T(C); TCT GAC CTC ACT CAG GAC CCT GCT GTC TCT GTG GCC TTG CGA CAG

G

A

C

T

V

R

I

T

C

Q

G

D

S

L

R

S

Y

A>

a

TRANSLATION OF 6B1 VL. SEQ [A] aaaa>

50	60	70	80	90
*	*	*	*	*
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA	A	P	V	A>
T	V	R	I	T
<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>

TRANSLATION OF 6B1 VL. SEQ [A] aaaa>

100 110 120 130 140

*	*	*	*	*				
AGC TGG TAC CAG CAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT	G	Q	K	P	V	L	V	Y>
S	W	Y	Q	Q	G	Q	Y	
<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	

TRANSLATION OF 6B1 VL. SEQ [A] aaaa>

150 160 170 180 190

*	*	*	*	*					
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC	G	K	N	N	R	P	S	G	S>
<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	<u>a</u>	

TRANSLATION OF 6B1 VL. SEQ [A] aaaa>

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Sequence Range: 1 to 324

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(v) 14F12 VL

Sequence Range: 1 to 321

27 | 43

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a TRANSLATION OF 14F12 VL. SEQ [A] a >

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Figure 3 Sequence of VH CDR3 of spiked clones derived from 1B2

PARENT (1-B2)	A R T G E Y S G Y D S S G V D V W
27-C1	A R T G E Y S G Y D T S G V E L W
27-D7	A R T R E Y S G H D S S G V D D W
27-E10	A R T G P F S G Y D S S G E D V R
27-H1	A R T E E Y S G Y D S S G V D V W
27-E2	A Q T R E Y T G Y D S S G V D V W
28-A11	A R T E E Y S G F D S T G E D V W
28-E12	A R T E E F S G Y D S S G V D V W
28-H10	A R T G E Y S G Y H S S G V D V R
31-G2	A R T E E F S G Y D S S G V D V W
30-B6	A R A G P F S G Y D S S G E D V R
30-E9	A R T G P F S G Y D S S G E D V W
30-F6	A R T E E F S G Y D S S G V D V W
30-D2	A R T G E Y S G Y D S S G E L V W
31-A2	A R T E E F S G Y D S T G E E V W
31-E11	A R T E E F S G Y D S S G V D V W
31-F1	A R T G E Y S G Y D S S G E D V W

Differences from 1B2 VH CDR3 are in bold.

Figure 4 Sequence of VT37 VL of antibody cross-reactive between TGFbeta1 and TGFbeta2

Sequence Range: 1 to 327

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	10	20	30	40	
*	*	*	*	*	
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG					
S S E L T Q D P A V S V A L G Q>					
—a—a—a—a TRANSLATION OF VT37-VL [A]—a—a—a—a->					
	50	60	70	80	
*	*	*	*	*	
ACA GTT AGG ATC ACT TCC CAA GGA GAC AGT CTC AGA AGC TAT TAC ACA					
T V R I T S Q G D S L R S Y Y T>					
—a—a—a—a TRANSLATION OF VT37-VL [A]—a—a—a—a->					
	100	110	120	130	140
*	*	*	*	*	*
AAC TGG TTG CAG CAG AAG CCA GGA CAG CCC CCT CTA CTT GTC GTC TAT					
N W F Q Q K P G Q P P L L V V Y>					
—a—a—a—a TRANSLATION OF VT37-VL [A]—a—a—a—a->					
	150	160	170	180	190
*	*	*	*	*	*
GCT AAA AAT AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC					
A K N K R P S G I P D R F S G S>					
—a—a—a—a TRANSLATION OF VT37-VL [A]—a—a—a—a->					
	200	210	220	230	240
*	*	*	*	*	*
AGC TCA GGA AAC ACA GCT TCC TGC ACC ATC ACT GGG CCT CAG GCG GAA					
S S G N T A S L T I P G A Q A E>					
—a—a—a—a TRANSLATION OF VT37-VL [A]—a—a—a—a->					
	250	260	270	280	
*	*	*	*	*	
GAT GAG GCT GAC TAT TAC TGT CAT TCC CGG GAC AGC AGT GGT AAC CAT					

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D E A D Y C H S R D S S G N H>
a a a a TRANSLATION OF VT37-VL [A] a a a a
290 * * 300 * * 310 * * 320 *
G T G C T T G C G C G A G G A C C A A G C T G A C C G T C C T A G G T
V L F G G T K L T V I G>
a a TRANSLATION OF VT37-VL [A] a a a>

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Figure 5. vh cassette 2

DNA sequence of heavy chain VH leader from intermediate vectors

With 6 enzymes: HINDIII SFI I PSTI BSTEII BAMHI ECOLI

March 30, 1994 09:50

H
I
N
D
I
I
I
aagcttgcggccaccatggactggacctggcgcggtgtttgacatgtctggccgtggccact
1-----+-----+-----+-----+-----+-----+ 60
ttcqaacggcggtggtaacctgacatggaccggcgacaaaaacggacgagggcaccgggaga

a K L A A T M D W T W R V F C L L A V A P -

S P B
F S E
I t I
I I Y
qqqqccccacagecagggtgcacatggcaggctgggtggcaagggaccacggtcaccgtct
61-----+-----+-----+-----+-----+-----+ 120
ccccgggtgtggteggtecacgttgcgtcaggccacgggttcctggtgccagggtggcaga

a G A H S Q V L Q Q S G A K G P R S P S -

B E
a c
m o
H R
I I
cctcagggtgagtggatccgaattc
121-----+-----+-----+ 144
ggagttccactcaccttaggttaag

a P Q V S G S E F -

Enzymes that do cut:

BamHI BstEII EcoRI HindIII PstI SfiI

Enzymes that do not cut:

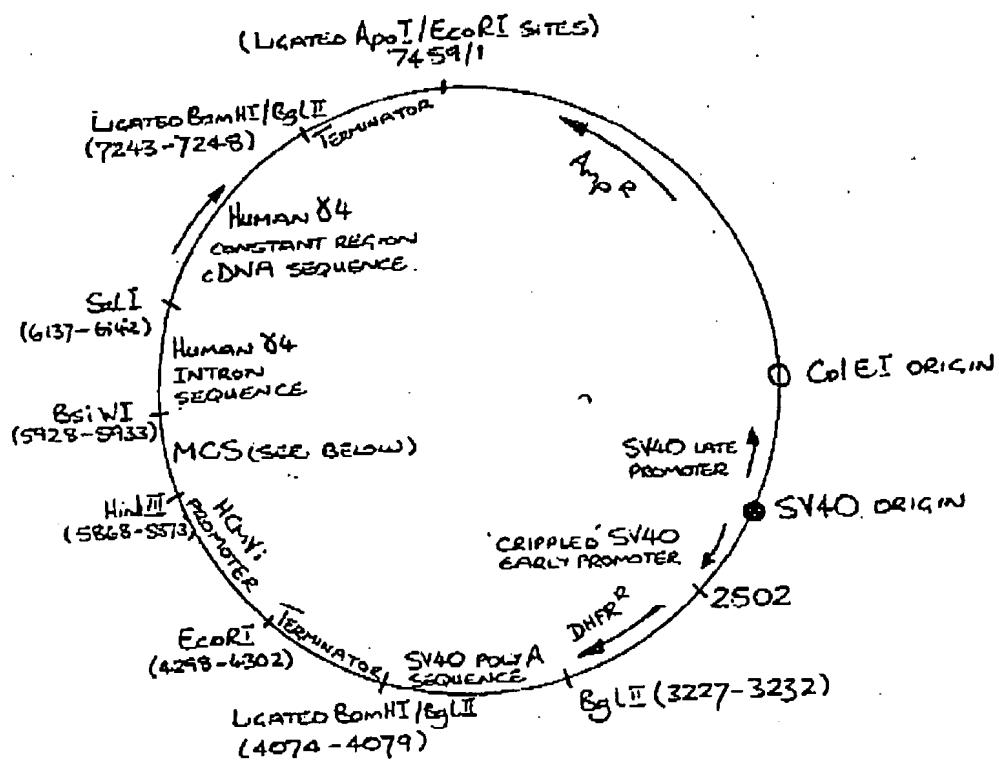
NONE

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Figure 6 pG4D100

Map

MAP OF pG4D100 (NOT TO SCALE)



MCS: ^{5'} HindIII - PacI - BamHI - (XbaI) - (PmlI) - (NheI) - AscI -
(BshII) - KhoI - PmeI - BsiWI ^{3'}

NOTE: THOSE R SITES SHOWN IN BRACKETS ARE NOT UNIQUE.

34183

Figure 7

v1cassetteCAT 1

DNA coding for v1 leader including intron. ApaLI site in the leader

with 5 enzymes: HINDIII SACI APALI XbaI BAMHI

June 16, 1994 15:44

H
 i
 n
 d
 I
 I
 I
 aagtttcgcacatggatggagctgtataatcttttggtagaaacagctacagg + 60
 1 -----+-----+-----+-----+-----+-----+-----+
 ttcgaagcggtggtaacctacctcgacatagtagaygagaagaaccatcggttgcgtatgc
 M G W S C I I L F L V A T A T
 taaggggctcacagtagcaggcttgaggcttgacatataatgggtgacaatgacatcc + 120
 61 -----+-----+-----+-----+-----+-----+-----+
 atccccggaggtgtcatcgccaaactcagacctgtatatacccaactgttactgtagg
 A
 P
 a
 L
 I
 S
 a
 c
 I
 actttgccttttcacacagggtgtgcactcggacattggagtcacccaggatccacaca + 180
 121 -----+-----+-----+-----+-----+-----+-----+
 tgaaaacggaaayagaggtgtccacacgtggctgtaaactcgagtggtcaqagggttgt
 G V K S D T E L
 X
 h
 o
 I
 B
 a
 m
 H
 I
 aaactcgagctgaaaacgtgagttagaatttaactttgccttcacattggatcc + 234
 181 -----+-----+-----+-----+-----+-----+-----+
 ttccatgttcacttttcactcatctttaatttggaaaacgaaaggagttacctagg

Enzymes that do cut:

Analyst BamHI HindIII SacI XbaI

Enzymes that do not cut:

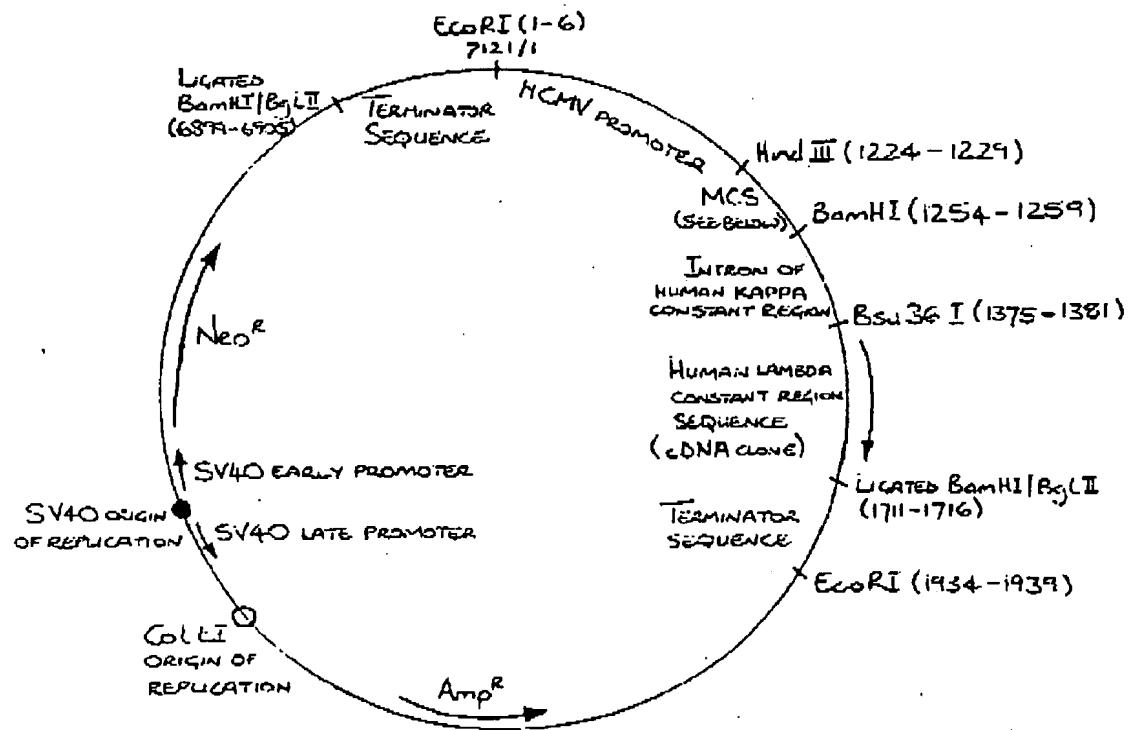
NONE

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Figure 8 pLN10

Map

MAP OF pLN10 (NOT TO SCALE)



MULTIPLE CLONING SITE (MCS)

⁵ Hind III - (Sph I) - (Pst I) - Sal I - Xba I - Bam HI ^{3'}

1224

1259

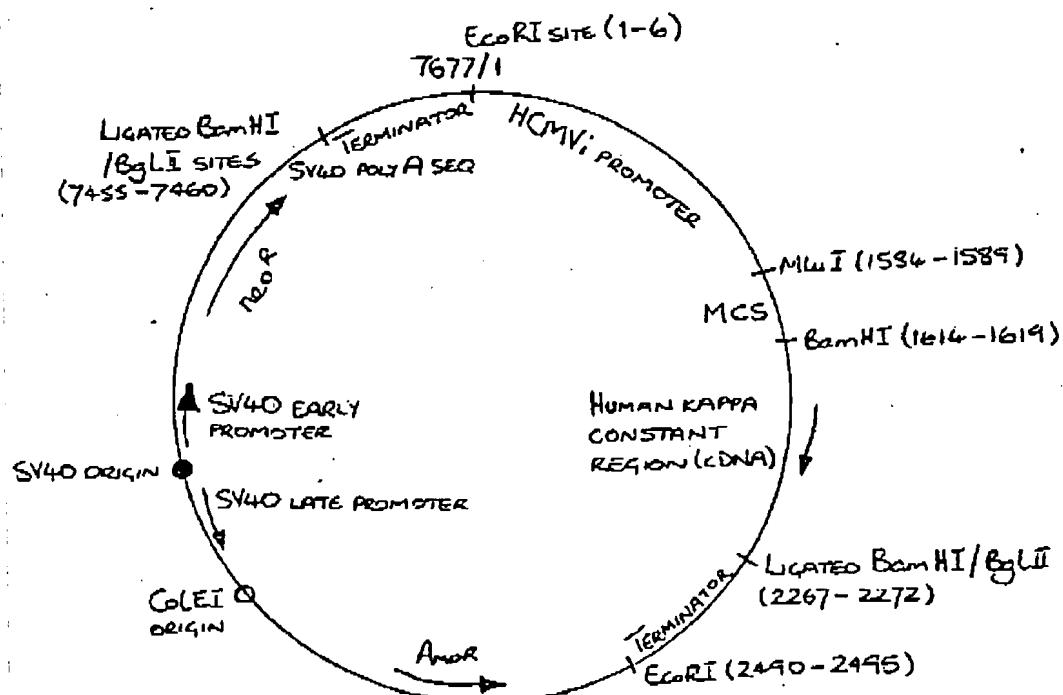
NOTE: RESTRICTION SITES IN BRACKETS ARE NOT UNIQUE.

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Figure 9 pKN100

Map

MAP OF pKN100 (NOT TO SCALE)



MCS: ^{5'} MluI - (Avr.I) - HindIII - (SphI) - (PstI) - SalI - XbaI - BamHI ^{3'}

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Fig 10 Neutralisation of TGF β 2 by scFv Antibodies

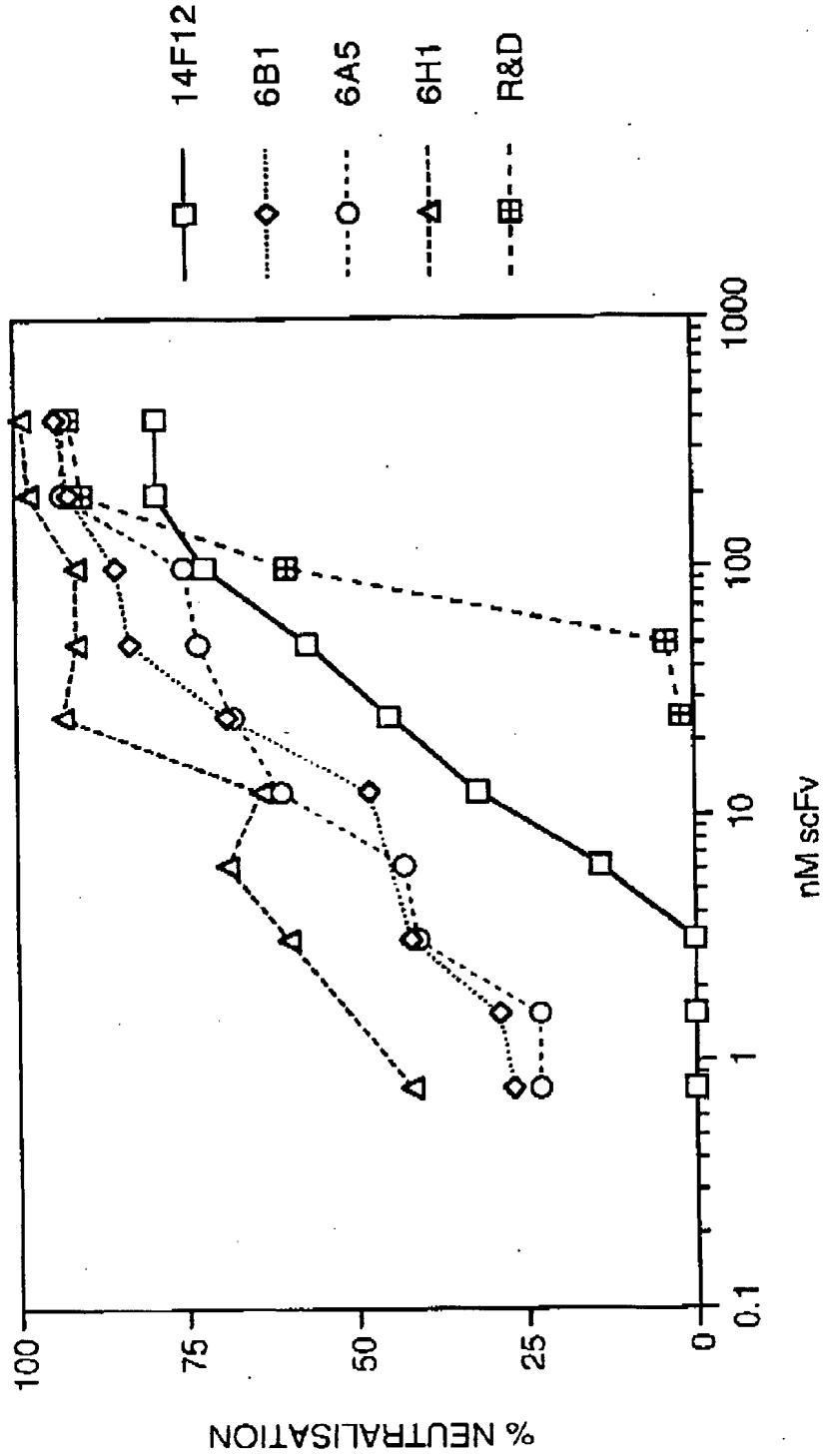
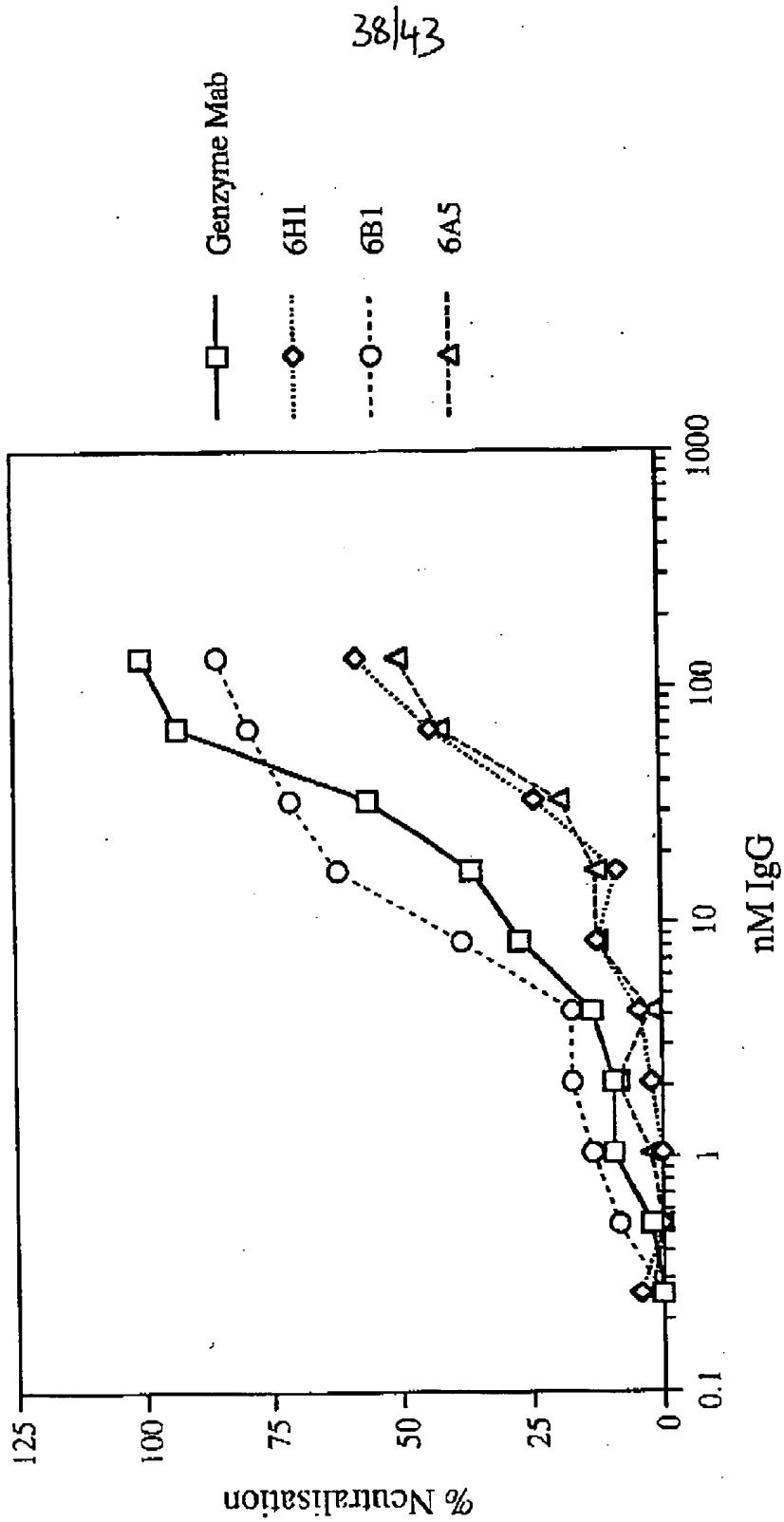
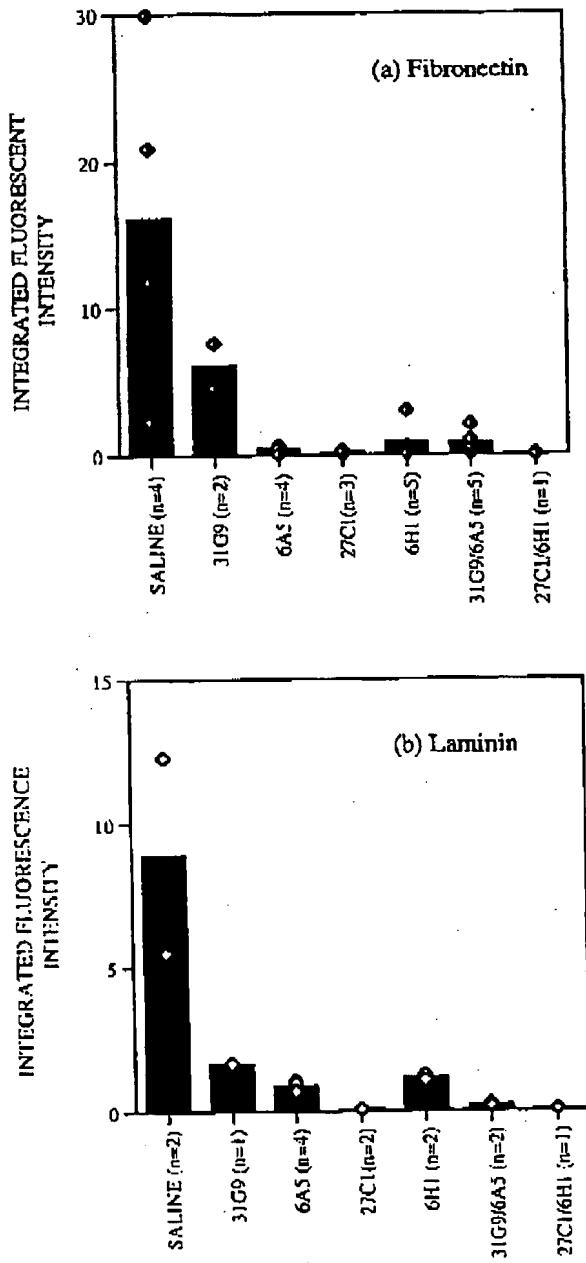


Fig 11 Neutralisation of TGF β_2 by Whole IgG4 Antibodies



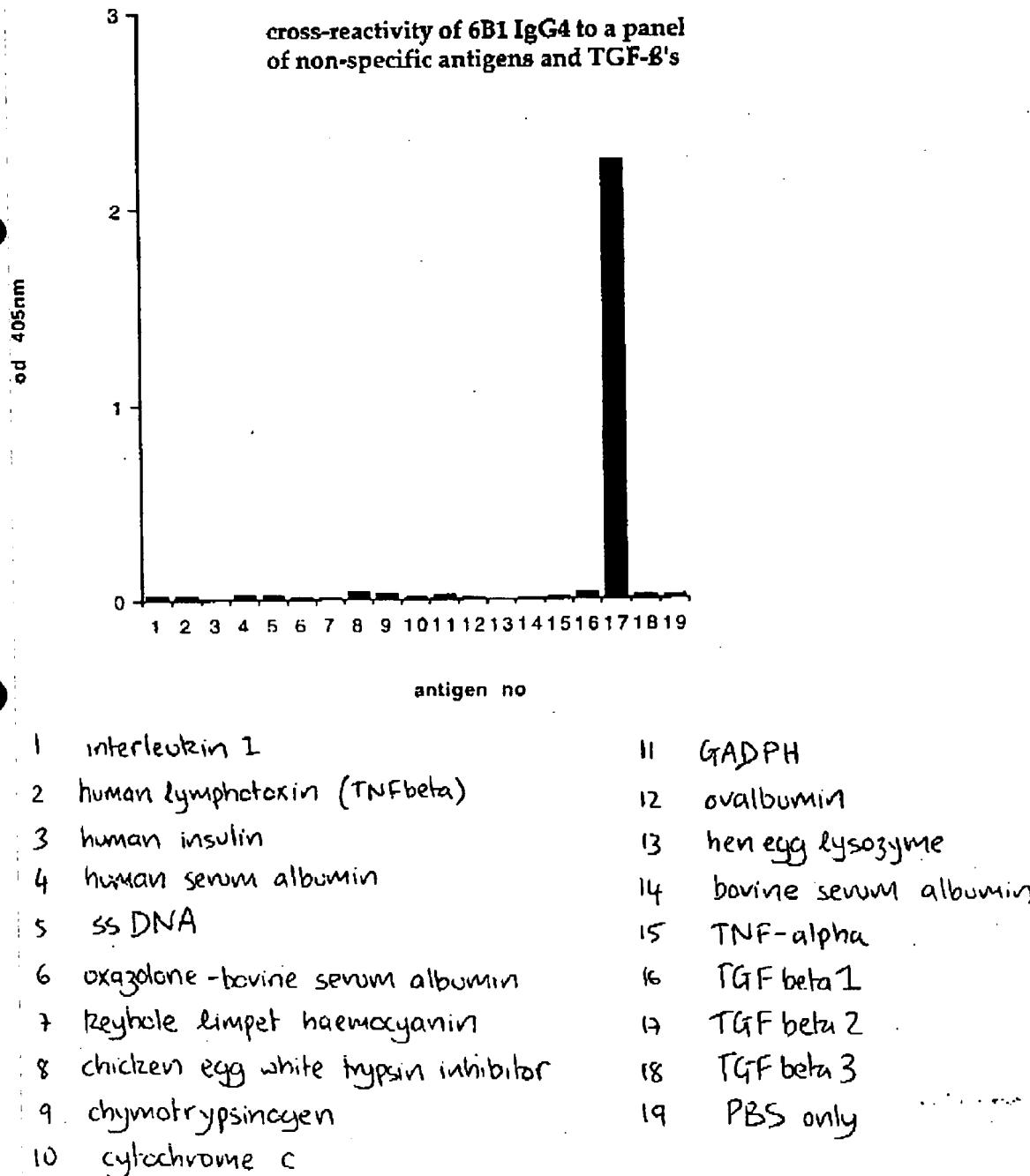
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Figure 12.
Scatter plots of individual animal data points. Bar graph is the
mean of the group.



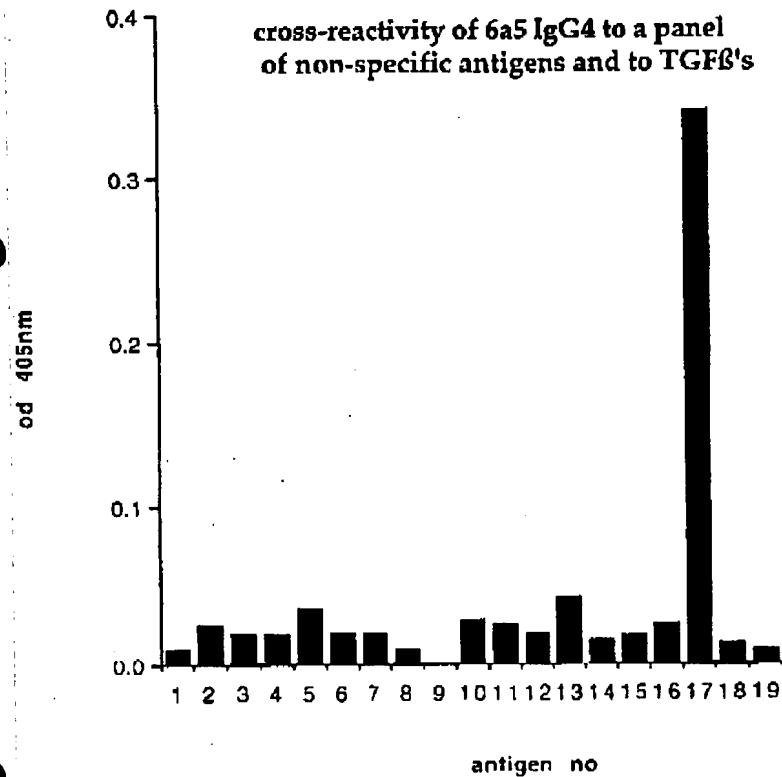
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Fig. 13(a)



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Fig. 13 (b)



For both Fig 13 (a) + (b). Antigens 1to15 were used for coating the plate at a concentration of 10 μ g/ml in PBS.

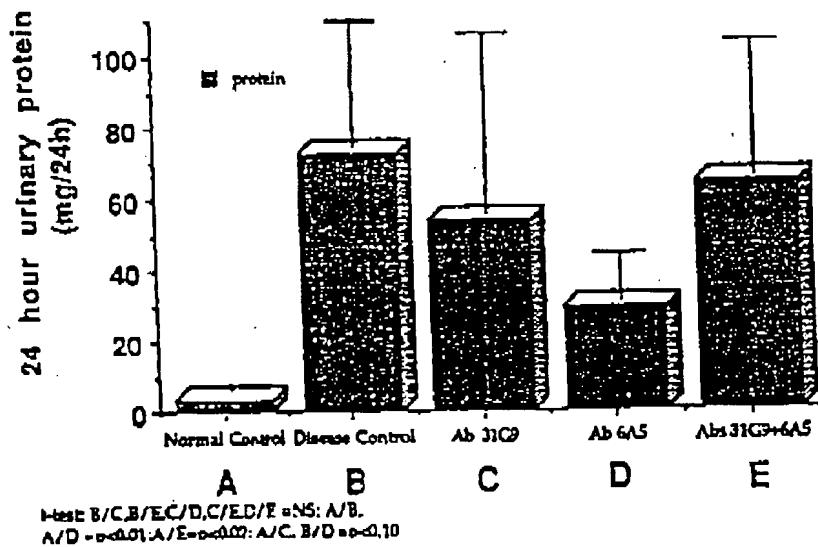
The TGFbetas were coated at 0.2 μ g/ml in PBS.

Coating was performed at 4°C overnight.

100 μ l of each antigen was used per well and duplicates of each antigen for each IgG to be tested. IgG samples were incubated with the coated antigens at 37°C for 2 hours after blocking with 2% normal - PBS. The labelled second antibody was a mouse anti-human Fc, alkaline phosphatase conjugated and the substrate used to detect bound second antibody was PNPP at 1 mg/ml with the absorbance read at 405 nm.

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FIGURE 14



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FIGURE 15

